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AWARD NUMBER: DAMD17-03-1-0522

TITLE: Constitutive Activation of NF-κB in Prostate Carcinoma Cells Through a Positive Feedback Loop: Implication of Inducible IKK-Related Kinase (IKKi)

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REPORT DATE: August 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* 01-08-2006	**2. REPORT TYPE** Annual	**3. DATES COVERED** *(From - To)* 1 Aug 2005 – 31 Jul 2006
4. TITLE AND SUBTITLE Constitutive Activation of NF-κB in Prostate Carcinoma Cells Through a Positive Feedback Loop: Implication of Inducible IKK-Related Kinase (IKKi)		**5a. CONTRACT NUMBER** **5b. GRANT NUMBER** DAMD17-03-1-0522 **5c. PROGRAM ELEMENT NUMBER**
6. AUTHOR(S) Irina V. Budunova, M.D., Ph.D. E-Mail: i-budunova@northwestern.edu		**5d. PROJECT NUMBER** **5e. TASK NUMBER** **5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Northwestern University Evanston, Illinois 60208-0110		**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)** **11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The overall goal of this project is to understand the role of inducible IKK-related kinase IKKi in constitutive activation of anti-apoptotic transcription factor NF-κB prostate carcinoma (PC) cells.

During FY03 we developed the optimal conditions for prostate cell PC cell stable infection by lentiviruses; regimens for selection of infected cells; and enrichment of cells co-infected with yellow fluorescent protein (YFP) by FACS. We also developed a test for monitoring of growth of live cell cultures infected with YFP using fluorescent plate reader. We continued the development of the conditions for IKKi immunoprecipitation to reveal the content of IKKi native complexes in PC cells. We assessed the biological effect of IKKi on prostate carcinoma cells. We found that IKKi in LNCaP prostate cells significantly increased their growth in vitro, increased their tumorigenicity. Our experiments indicated that IKKi affected the transcriptional activity of androgen receptor. These results suggest an important role of IKKi in the transition of prostate cells to androgen-independent growth.

The results of our studies have been presented at the local and national meetings, one manuscripts is published, one is in press, one is under revision, and fourth is under preparation.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	32	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Introduction

The overall goal of this project is to understand the role of inducible IKK-related kinase IKKi in constitutive activation of anti-apoptotic transcription factor NF-κB prostate carcinoma (PC) cells.

During FY03 the major directions of our work were (i) to assess the biological effects of IKKi overexpression in PC cells stably infected with lentiviruses harboring w.t. IKKi, and (ii) to continue developing the technical conditions for cell lysis and IKKi immunoprecipitation to reveal the content of IKKi native complexes in PC cells.

The results of our studies have been presented at the local and national meetings, one manuscripts is published, one is in press, one is under revision, and fourth is under preparation. The following describes the progress made in this year.

Body

During current year we specifically focused on the experiments pertinent to the tasks 2 and 3.

Task 2.

Our pilot experiments indicated that the transcriptional activity of androgen receptor was increased in LNCaP cells expressing IKKi (see below). Taking into consideration, that IKKi has nuclear localization in normal and cancer prostate glands and in PC cells in vitro (see progress report for FY02), we started to evaluate the co-localization of IKKi and androgen receptor in prostate samples and in PC cell cultures using rabbit polyclonal antibody against IKKi (ProSci, Poway, CA) and mouse monoclonal antibody against AR (Santa Cruz Biotechnology, Pasadena, CA), and EnVision^{TM} DoubleStain kit (DakoCytomation). In the limited immunostaining experiments we found that AR and IKKi are co-expressed in LNCaP-IKKi cells that express endogenous AR and exogenous IKKi. We also started to assess the correlation between AR and IKKi expression in prostate samples. The staining will be reviewed and scored by Dr. Yang, an Associate Director of Pathology Core at R. Lurie Cancer Center, who has an extensive experience in quantitative analysis of PC marker expression. The tissue sections analyzed by Dr. Yang will be scanned, and images will be converted to digital files using the ACIS II system (ChromaVision Medical Systems, Inc.) for quantitative analysis.

Task 3.

In FY02 we have generated several independent clones of LNCaP and PC3 cell lines stably expressing IKKi w.t. and IKKi d.n. mutant under CMV promoter to study the mechanism of IKKi transport to the nucleus and to assess the effect of IKKi localization on its function. These lines were generated using commercially available lentiviral system (Invitrogen Corporation) and were characterized by the increased number (up to 85-90%) of IKKi-expressing cells in the bulk cultures. We continued to developing the technical conditions for cell lysis and IKKi immunoprecipitation to reveal the content of IKKi native complexes in PC cells.

In addition, in the current year we have characterized the effect of IKKi in LNCaP cells on basal and inducible NF-kB activity. It was found recently that phosphorylation of major NF-kB subunit p65 at Ser536 is important for NF-kB activity and strongly depends on IKKi (Adli and Baldwin, 2006). Thus, to assess NF-kB activity in prostate cells we evaluated the level of IkBa phosphorylation and the level of p65 phosphorylation at Ser536 (both antibodies are Cell Signaling Technology, Beverly, MA). We found that both basal and inducible level of NF-kB were increased in LNCaP-IKKi cells, and that p65 phosphorylation was more significant in IKKi-expressing LNCaP cells treated with standard NF-kB inducers such as IL-1 and TNF-a.

Further, we started to analyze the effect of IKKi on prostate cell growth and tumorigenicity. To track live LNCaP-IKKi cells in vitro and in vivo we have generated LNCaP clones co-expressing w.t. IKKi and yellow fluorescent protein (YFP). LNCaP cells infected with the empty vector (LNCaP-V) or with the YFP-expressing lentivirus (LNCaP-YFP) were used as control. We took advantage of YFP expression in the LNCaP-GR-YFP cells and developed the assay to measure the actual number of cells/well by fluorescent plate-reader.

Using these cell lines we showed that IKKi significantly increased LNCaP cells growth in monolayer. Importantly, IKKi-expressing LNCaP cells were characterized by the increased tumorigenicity assessed by anchorage-independent growth (colony formation assay in soft agar). Using PARP cleavage assay, we also found that IKKi-expressing LNCaP cells are less sensitive than control LNCaP cells to apoptosis induced by TNF-a.

Taking into consideration our initial finding that androgen-independent PC cells express high amount of IKKi, we started to study the effect of IKKi on androgen-mediated

signaling and the tolerance to androgen ablation using LNCaP-IKKi cells. Our pilot experiments indicated that the transcriptional activity of androgen receptor was increased in LNCaP cells expressing IKKi, and that they grew better than LNCaP-vector transfected cells in the medium with char coal stripped serum. Thus, our results suggest an important role of IKKi in the transition of prostate cells to androgen-independent growth.

Finally, taking into consideration the newly recognized association of prostate inflammation and prostate cancer that offers one of the greatest opportunities for preventing malignant conversion (De Marzo et al., 2003, Platz and De Marzo, 2004) we continued to study the cross-talk between pro-inflammatory signaling mediated by IKKi and NF-kB and anti-inflammatory signaling mediated by glucocorticoid receptor in prostate carcinoma cells. In FY02 we found that glucocoticoids inhibit IKKi function. During FY03 we showed that glucocorticoid receptor is lost in @ 80% of prostate carcinomas, and acts as a tumor suppressor in prostate cells.

Key Research Accomplishments

- We have developed protocols for :
    - optimal PC cell stable infection by lentiviruses;
    - optimal regimens of selection of transfected cells;
    - enrichment of cells co-infected with YFP by FACS.
- We provided consultations on the lentiviral infection of epithelial cells and post-infection selection to the numerous researchers at Northwestern University.
- w.t. IKKi increased both basal and constitutive NF-kB activity in LNCaP cells.
- w.t. IKKi increased LNCaP cell growth in monolayer.
- w.t. IKKi increased LNCaP cell tumorigenicity/anchorage-independent growth assessed by soft agar colony formation.
- IKKi positively affects androgen receptor transactivation potential in prostate carcinoma cells.
- Glucocorticoids inhibit IKKi expression in androgen-independent prostate carcinoma cells DU145.
- We found that the expression of glucocorticoid receptor (GR) is dramatically decreased in @ 80% of prostate carcinomas. We also showed that activated GR functions as a tumor suppressor in prostate cells, and inhibits multiple signaling pathways and transcriptional factors involved in proliferation and transformation including NF-kB.

Reportable outcomes:

Abstracts :

1. Yemelyanov A., Czwornong J., Chebotaev D., Karseladze A., Yang X., Budunova I. Expression and function of glucocorticoid receptor in prostate carcinomas and PC cells. . Keystone Symposium: Hormonal regulation of tumorigenesis. February 20-25, 2005, Monterey, CA, p. 43.

2. Yemelyanov A., Czwornong J., Chebotaev D., Karseladze A., Yang X., Budunova I. Expression and function of glucocorticoid receptor in prostate carcinomas and PC cells. The Chicago Signal Trunsduction Symposium, May 2005, Chicago, IL.

3. A. Gasparian, N. Gasparian, A. Yemelyanov, D. Chebotaev, F. Kisseljov, and I. Budunova. Targeting NF-κB in prostate carcinoma cells: comparative analysis of proteasome and IKK inhibitors. Keystone Symposium: NF-kB: 20 years on the road from biochemistry to pathology. March 23-28, 2006, Banff, Alberta, Canada, p. 53.

4. A. Yemelyanov, A. Gasparian, P. Lindholm, L. Dang, F. Kisseljov, A. Karseladze, and I. Budunova. Effects of IKK inhibitor PS1145 on NF-κB function, proliferation, apoptosis and invasion activity in prostate carcinoma cells. 28, 2006, Banff, Alberta, Canada, p. 63.

5. Yemelyanov A., Czwornong J., Chebotaev D., Karseladze A., Yang X., Budunova I. Decreased expression of glucocorticoid receptor in prostate carcinomas and its anti-tumorigenic activity in PC cells *in vitro*. Proceedings of AACR *47*, 2006 (abstract # 5335).

Publications.

1. Yemelyanov A., Gasparian A., Lindholm P., Dang L., Pierce J., F. Kisseljov, A. Karseladze, Budunova I. Effect of IKK inhibitor PS1145 on NF-kappaB function, proliferation, apoptosis, and invasion activity in prostate carcinoma cells. Oncogene, 2006, 25(3):387-98.

2. Yemelyanov A., Czwornog J., Chebotaev D., Karseladze A., Kulevitch E., Yang X., Budunova I. Tumor suppressor effect of glucocorticoid receptor in prostate. Oncogene, 2006, in press.

3. T. Nelius, S. Filleur, A. Yemeyanov, I. Budunova, E. Shroff , Y. Mirochnik, A. Aurora, D. Veliceasa and O.V. Volpert. The pro-apoptotic and anti-angiogenic effects of androgen in the in vivo model of prostate cancer. Under revision.

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Conclusions

Our data provide experimental evidence that IKKi could be involved in the regulation of NF-kB activity in PC cells through a positive feedback loop. IKKi is highly expressed in androgen-independent malignant PC cell lines. The introduction of w.t. IKKi into androgen-dependent LNCaP prostate cells significantly increased their growth in vitro, increased their tumorigenicity (assessed in clonogenic assay in vitro), and protected LNCaP cells from the induced apoptosis. Our pilot experiments also indicated that the transcriptional activity of androgen receptor was increased in LNCaP-IKKi cells. Taking into consideration, that IKKi is expressed in prostate glands and that nuclear IKKi expression was increased in human prostate carcinomas in comparison to BPH samples (see progress report for FY02), these recent results suggest an important role of IKKi in the transition of prostate cells to androgen-independent growth and malignant conversion of prostate tumors.

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Appendices

2. Yemelyanov A., Gasparian A., Lindholm P., Dang L., Pierce J., F. Kisseljov, A. Karseladze, Budunova I. Effect of IKK inhibitor PS1145 on NF-kappaB function, proliferation, apoptosis, and invasion activity in prostate carcinoma cells. Oncogene, 2006, 25(3):387-98.

2. Yemelyanov A., Czwornog J., Chebotaev D., Karseladze A., Kulevitch E., Yang X., Budunova I. Tumor suppressor effect of glucocorticoid receptor in prostate. Oncogene, 2006, in press.

Oncogene (2005), 1–12

www.nature.com/onc

ORIGINAL ARTICLE

Effects of IKK inhibitor PS1145 on NF-κB function, proliferation, apoptosis and invasion activity in prostate carcinoma cells

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A key antiapoptotic transcription factor, nuclear factor kappa-B (NF-κB), is known to be critically important for tumor cell growth, angiogenesis and development of metastatic lesions. We and others showed previously that NF-κB transcription factor was constitutively activated in androgen-independent prostate carcinoma (PC) cell lines due to the upregulated activity of inhibitor of NF-κB kinases (IKK). In this work, using luciferase assay, electrophoretic mobility shift assay and Northern blot analysis of expression of endogenous κB-responsive genes, we demonstrate that a novel highly specific small-molecule IKK inhibitor, PS1145, efficiently inhibited both basal and induced NF-κB activity in PC cells. We found that PS1145 induced caspase 3/7-dependent apoptosis in PC cells and significantly sensitized PC cells to apoptosis induced by tumor necrosis factor alpha. We also showed that PS1145 inhibited PC cell proliferation. Effects of PS1145 on proliferation and apoptosis correlated with inhibition of interleukin (IL)-6, cyclin D1, D2, inhibitor of apoptosis (IAP)-1 and IAP-2 gene expression and decreased IL-6 protein level. In addition, we found that incubation with PS1145 inhibited the invasion activity of highly invasive PC3-S cells in invasion chamber assay in a dose-dependent manner. Overall, this study provides the framework for development of a novel therapeutic approach targeting NF-κB transcription factor to treat advanced PC.
Oncogene advance online publication, 19 September 2005; doi:10.1038/sj.onc.1209066

Keywords: prostate carcinoma; NF-κB; IKK; apoptosis; invasion

Introduction

One of the contributing factors to high mortality rate from prostate cancer is the extreme resistance of malignant prostate cells to apoptosis induced by radio- and chemotherapy. Thus, the specific induction of apoptosis in prostate carcinoma (PC) cells could play a strategic role for PC treatment.

Nuclear factor kappa-B (NF-κB) transcription factor mediates one of the central signaling pathways, protecting cells from apoptotic death (Karin and Lin, 2002; Kucharczak *et al.*, 2003). NF-κB also regulates tumor development through transcriptional regulation of a wide variety of genes that encode antiapoptotic proteins, cell cycle-related proteins, proteins involved in angiogenesis, invasion and metastasis (Ghosh and Karin, 2002; Karin *et al.*, 2002; Shishodia and Aggarwal, 2004).

The active NF-κB complex is a homo- or heterodimer composed of proteins from the NF-κB/Rel family: NF-κB1 (p50/105), NF-κB2 (p52/100), RelA (p65), RelB and c-Rel (Verma *et al.*, 1995; Baldwin, 1996). In nonstimulated cells, NF-κB resides in the cytoplasm in a complex with the inhibitor protein, collectively called IκB. Several inhibitor of nuclear factor kappa-B (IκB) proteins have been identified, including IκBα, IκBβ and IκBε, and proteins p105 and p100, the precursor molecules for NF-κB proteins p50 and p52, respectively (Verma *et al.*, 1995; Whiteside and Israel, 1997). Most agents that activate NF-κB employ a common pathway that involves the phosphorylation of the two N-terminal serines in IκB molecules IκBα, IκBβ, IκBε, and the subsequent ubiquitination and degradation of IκB proteins by the 26S proteasome (Whiteside and Israel, 1997). Signal-induced phosphorylation of IκB is executed by a large 900 kDa IκB kinase (IKK) complex, containing two major IκB kinases (IKK) IKKα and IKKβ, as well as several scaffolding proteins (Karin and Ben-Neriah, 2000).

There is mounting evidence that constitutive NF-κB activation is a common feature of a variety of hematological and solid tumor cell lines and tumors (Rayet and Gelinas, 1999; Karin *et al.*, 2002), and that constitutive activation of NF-κB suppresses the susceptibility of tumor cells to apoptosis induced by radio- and chemotherapy. We and others showed previously that NF-κB was constitutively activated in androgen-independent PC cell lines due to the constitutive upregulated activity of IKK kinases (Chen and Sawyers, 2002; Gasparian *et al.*, 2002; Suh *et al.*, 2002; Zerbini *et al.*, 2003). NF-κB inhibition with a IκBα super-repressor in

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PC cells led either to apoptosis or to sensitization to apoptosis induced by tumor necrosis factor alpha (TNF-α) and some other treatments (Herrmann *et al*., 1997; Muenchen *et al*., 2000; Gasparian *et al*., 2002). Most importantly, inhibition of NF-κB with an IκBα super-repressor in PC cells suppressed both growth and development of metastatic lesions by those cells *in vivo* when they were injected into prostate ortotopically (Huang *et al*., 2001). Overall, these data suggest that NF-κB signaling pathway is critically important for PC cell growth and the development of metastases in animal models. Thus, NF-κB represents an important target for PC treatment, especially when NF-κB inhibition is used in combination with other proapoptotic chemotherapeutical drugs.

The results of immunostaining of human PCs for major NF-κB protein RelA/p65 are in line with the data obtained in PC cells *in vitro*. We and others showed that p65 has nuclear localization in a significant number of epithelial cells in prostate tumors, especially in hormone-refractory metastatic PCs (Gasparian *et al*., 2002; Ross *et al*., 2004; Shukla *et al*., 2004; Sweeney *et al*., 2004). Nuclear localization of p65 strongly suggests that NF-κB is activated in human PCs, and that constitutive activation of this key antiapoptotic factor could significantly contribute to the resistance of hormone-refractory PCs to apoptosis induction during chemo- and radiotherapy.

There are several pharmacological approaches to target NF-κB. They include repression of NF-κB transactivation potential, stabilization of IκB inhibitors by proteasome inhibitors and, more recently, inhibition of upstream IKK kinases (Karin, 2004). The unique properties of IKKβ among other serine–threonine kinases allowed successful development of specific IKKβ inhibitors at Millennium Pharmaceuticals Inc. (Hideshima *et al*., 2002; Castro *et al*., 2003; Lam *et al*., 2005) and other companies (Burke *et al*., 2003; Kishore *et al*., 2003; Ziegelbauer *et al*., 2005). In the presented work, we studied the effect of the small-molecule IKK inhibitor PS1145 on the status of NF-κB in PC cells, PC cell growth, sensitivity of PC cells to apoptosis and on the invasion capability of PC cells. We demonstrated that PS1145 efficiently inhibited both basal and induced NF-κB activity in PC cells. PS1145 induced apoptosis in PC cells and significantly sensitized PC cell lines to TNF-α-induced apoptosis in a caspase 3/7-dependent manner. In addition, we found that preincubation with PS1145 inhibited PC cell growth and the invasion activity of highly invasive PC3-S cells in invasion chamber assay.

Results

Expression of activated IKKs in PCs

Our previous studies as well as data by others revealed that androgen-independent PC cells maintain the high level of NF-κB basal activity due to constitutive IKK activation (Gasparian *et al*., 2002). To extend our *in vitro* observations, we performed immunostaining of 10 high-grade PCs (Gleason score 7–9) with Ab against IKKα/β phosphorylated at Ser176/180. It is known that activation of IKKα and IKKβ requires their phosphorylation at those specific serines in the activation loop of IKK kinases (Karin and Ben-Neriah, 2000; Huynh, 2000). As shown in Figure 1, activated IKKα/β were strongly expressed in the cytoplasm of epithelial cells in PCs. This finding suggests that IKK kinases are indeed constitutively active in prostate cells in tumors and correlates well with nuclear localization of p65/RelA and NF-κB activation in PCs previously described in our work and in other publications (Gasparian *et al*., 2002; Ross *et al*., 2004; Shukla *et al*., 2004; Sweeney *et al*., 2004).

PS1145 inhibited basal and induced IκBα phosphorylation and NF-κB activity in androgen-independent PC cell lines

We and others have shown that NF-κB is constitutively activated in androgen-independent PC cell lines due to the constitutive upregulated activity of IKK kinases. The IκBα protein, a key substrate for IKKα/IKKβ kinase complex, is constitutively phosphorylated in PC3 and DU145 cells (Gasparian *et al*., 2002). Despite the high constitutive level of NF-κB activity, androgen-independent PC cells appeared to be highly sensitive to diverse NF-κB inducers (Gasparian *et al*., 2003). For example, DU145 cells are highly responsive to TNF-α and lipopolysaccharide (LPS), while PC3 cells are highly responsive to LPS and TPA, but not to TNF-α. Based on these findings, we selected different NF-κB inducers to study the effect of PS1145 on NF-κB activation in PC3 and DU145 cells.

To evaluate the effect of the IKK inhibitor PS1145 on phosphorylation of IκBα, we performed Western blot analysis using whole-cell proteins from DU145 or PC3 cells treated with PS1145 at concentrations 0.5–20 μM for 2–24 h. We found that the PS1145 effect was especially pronounced when cells were incubated with this IKK inhibitor at concentrations 10–20 μM (Figure 2a and data not shown). The significant decrease of IκBα constitutive phosphorylation was revealed 12–16 h after treatment in both DU145 and PC3 cells (Figure 2a and b and data not shown). Furthermore, IκBα phosphorylation was almost completely blocked when cells were incubated with PS1145 for 24 h (Figure 2a and b). However, in PC3 cells decrease in

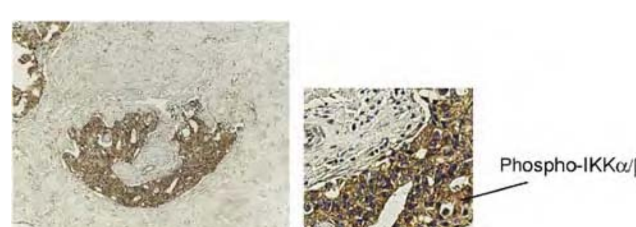

Figure 1 IKKα/β are phosphorylated in PCs. Immunostaining of PC (Gleason score 9) with antibodies against IKKα/β phosphorylated at Ser176/180 in IKK activation loop. Note the strong expression of phosphorylated IKKs in epithelial cells of PC.

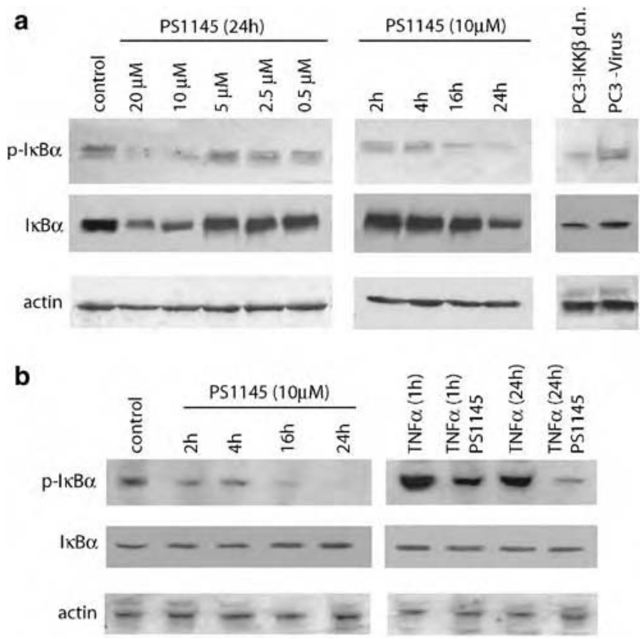

Figure 2 PS1145 inhibited basal and induced IκBα phosphorylation. (**a**) PC3 cells were treated with PS1145 at the indicated concentrations (0.5–20 μM) for 2–24 h. Far right lanes: PC3 cells stably infected with empty lentivirus or lentivirus expressing d.n. IKKβ mutant. (**b**) DU145 cells were treated with 10 μM PS1145 for 2–24 h. Far right lanes: DU145 cells were pretreated with PS1145 for 24 h and treated with TNF-α (7 ng/ml) for 1 h or treated with both agents for 24 h. Western blots containing whole-cell protein extracts (50 μg/lane) were probed with anti-IκBα and anti-phospho-IκBα antibodies or anti-actin Ab as a control.

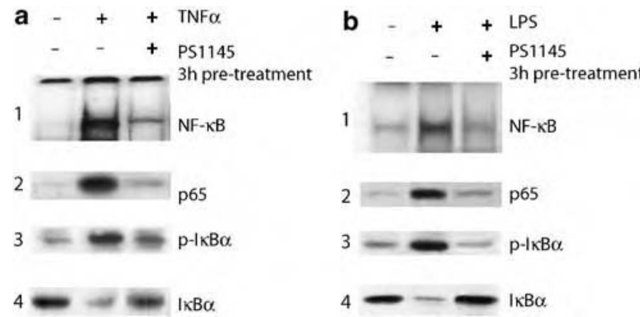

Figure 3 PS1145 inhibited NF-κB binding in PC cells. (**a**). Control and PS1145-pretreated (10 μM $\times$ 3 h) DU145 cells were treated with TNF-α (7 ng/ml) for 10 min. (**a.1**) Nuclear protein extracts were used to evaluate κB binding by EMSA; (**a.2**) Western blots containing nuclear protein extracts (20 μg/lane) were probed for expression of p65; (**a.3–a.4**) Western blots containing cytoplasmic protein extracts (20 μg/lane) were probed for expression of IκBα and IκBα-P. (**b**) Control and PS1145-pretreated (10 μM $\times$ 3 h) PC3 cells were treated with LPS (1.5 μg/ml) for 1 h. Procedures used in (**b.1–b.4**) are identical to those described in (**a.1–a.4**).

IκBα phosphorylation 24 h after PS1145 treatment coincided with inhibition of total IκBα expression at this time point (see below), suggesting that the relative level of inhibition of IκBα phosphorylation (ratio P-IκBα:total IκBα) in PC3 cells was similar after 16 and 24 h of treatment with PS1145.

PS1145 also strongly inhibited induced IκBα phosphorylation. Indeed, preincubation of PC cells with PS1145 for 3 h significantly inhibited IκBα phosphorylation induced by short treatments with different compounds such as TNF-α, LPS and TPA (Figure 3 and data not shown). In addition, we found a stable strong effect of long cell pretreatment with PS1145 (24 h) on TNF-α-induced IκBα phosphorylation in DU145 cells (Figure 2b). Importantly, the similar decrease in IκBα phosphorylation was observed in PC3 cells stably infected with lentivirus expressing IKKβ dominant-negative (d.n.) mutant (Figure 2a, last lane).

We have to mention that treatment of PC3 cells with PS1145 for 16–24 h as well as the transfection with exogenous IKKβ d.n. mutant has resulted in the decreased level of total IκBα. This reflects the dramatic downregulation of IκBα gene transcription in PC3 cells under this treatment (Figure 5b) and will be discussed later. PS1145 did not affect IκBα expression in DU145 cells (Figure 2b).

In our next experiments, we examined PS1145 effect on NF-κB DNA binding in PC cells. The effect of PS1145 on different steps of NF-κB activation was dose-dependent, and more pronounced when we used PS1145 at the concentration of 20 μM (data not shown). As shown in Figure 3, pretreatment of DU145 cells with PS1145 for 3 h strongly inhibited IκBα phosphorylation, delayed the degradation of IκBα and nuclear translocation of p65 induced by TNF-α, and accordingly significantly decreased the effect of TNF-α on κB DNA binding. Similarly, PS1145 inhibited IκBα phosphorylation, degradation, p65 nuclear translocation and NF-κB binding induced by LPS (Figure 3b) and TPA (data not shown) in PC3 cells.

To evaluate the effect of PS1145 on gene transcription, we used transient transfection of PC cells with a 5$\times$ κB luciferase reporter. To induce NF-κB-dependent transcription of reporter gene, PC cells were transfected with exogenous IKKβ or treated with the appropriate κB inducer: TNF-α (for DU145 cells) or LPS (for PC3 cells) for 24 h. NF-κB activity induced by TNF-α, LPS and exogenous IKKβ was strongly inhibited by PS1145 in both PC cell lines (Figure 4a and b). In addition, PS1145 significantly blocked basal NF-κB transcriptional activity in PC3 cells (Figure 4b).

To study the effect of PS1145 on the transcription of endogenous κB-responsive genes, we used Northern blot analysis of IκBα expression. IκBα gene has five κB sites in its promoter, and is tightly regulated by NF-κB in different cells (Ito *et al*., 1994). We showed previously that the level of steady-state IκBα expression directly correlated with the level of constitutive NF-κB activity in different PC cell lines (Gasparian *et al*., 2002). The results of Northern blotting demonstrated that treatment with PS1145 (20 μM) for 24 h blocked both basal and inducible expression of IκBα in PC cells (Figure 5a and b). The effect of PS1145 on IκBα expression was dose- and time-dependent (data not shown) with maximum IκBα expression blockage after 24 h exposure to PS1145.

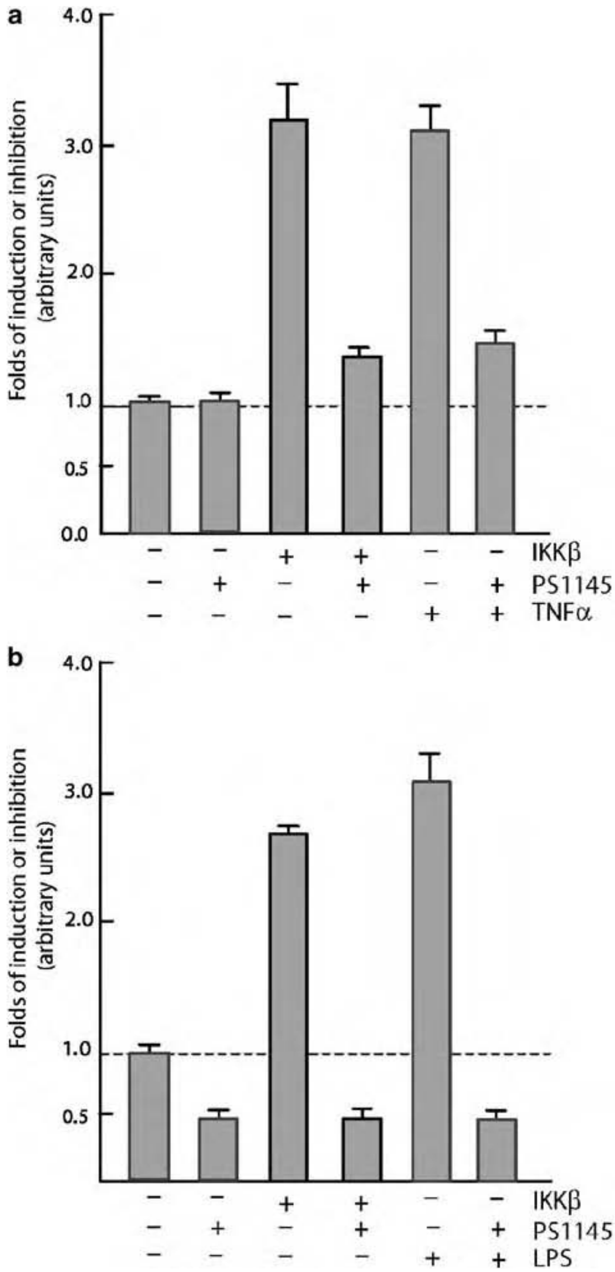

Figure 4 Effect of PS1145 on NF-κB transcriptional activity in PC cell lines. DU145 (**a**) and PC3 (**b**) cells were cotransfected with $\times$ 5 κB FL reporter, pRNL-null (RL), pcDNA and with w.t. IKKβ plasmids. Cells were treated with TNF-α (7 ng/ml), LPS (10 μg/ml), PS1145 (10 μM) or the combination PS1145 + TNF-α or PS1145 + LPS for 24 h. Luciferase activity was measured by dual luciferase assay. Data are shown as fold of inhibition or induction, calculated as FL/RL ratio for treated samples normalized against FL/RL ratio for control samples (controls are presented in the far left lanes in (**a**) and (**b**)).

Overall, the results of Northern blot analysis correlated well with the data obtained by Luciferase reporter assay and electrophoretic mobility shift assay (EMSA). We found that PS1145 strongly inhibited NF-κB activity in both PC cell lines irrespectively of the nature of the NF-κB inducer. Notably, the effect of PS1145 on basal NF-κB activity was revealed easier in PC3 cells despite

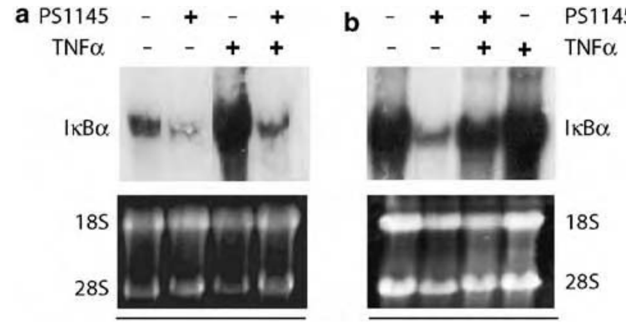

Figure 5 Northern blot analysis of IκBα expression in PC cells after PS1145 treatment. DU1145 (**a**) and PC3 cells (**b**) were treated with TNF-α (7 ng/ml), PS1145 (10 μM) or the combination PS1145 + TNF-α for 24 h. Northern blots (20 μg RNA/lane) were probed for expression of IκBα. Ethidium bromide gel staining (lower panels) was used for the verification of equal RNA loading.

the fact that PS1145 strongly inhibited basal IκBα phosphorylation in both PC cell lines.

PS1145 inhibited proliferation of DU145 cells

The effect of PS1145 on proliferation was assessed by several approaches in DU145 cells. As shown in Figure 6a, MTT test revealed 30–35% decrease in DU145 cell numbers 48–72 h after PS1145 treatment. BrdU labeling of DU145 cells confirmed the result of MTT test. The number of BrdU-positive cells (cells in S-phase) was decreased by $32 \pm 3.25\%$ in DU145 cell cultures treated with PS1145 for 72 h (Figure 6b). The inhibition of proliferation was further confirmed by the decrease of Ki67 protein expression known to be present in cells in G1, S, G2 and M phases, but not in G0 phase of the cell cycle (data not shown). Therefore, we have shown that PS1145 caused significant inhibition of proliferation in DU145 cells.

PS1145 induces apoptosis in DU145 cells and increases their sensitivity to TNF-α

Long-term exposure to PS1145 was toxic for DU145 cells. To evaluate the effect of PS1145 on apoptosis in these cells, we measured caspase 3/7 activity, and used Western blot analysis to assess poly-(ADP-ribose) polypeptide (PARP) cleavage. As shown in Figure 7a, treatment of DU145 cells with PS1145 for 48 h resulted in strong activation of caspase 3/7 in a dose-dependent manner. Western blot analysis of caspase 3/7-dependent PARP cleavage also demonstrated that PS1145 induced apoptosis in DU145 cells 48–72 h after the beginning of the treatment (Figure 7b and c). Importantly, PS1145 sensitized DU145 cells to TNF-α-induced apoptosis: PARP cleavage and caspase 3/7 activation (Figure 7b and c) were much more pronounced in DU145 cells treated with combination of TNF-α and PS1145. These data are in line with the previous observations that NF-κB protects different cells, including PC cells, against apoptosis induced by TNF-α, and that NF-κB blockage by different genetic approaches results in cell sensitization to TNF-α (Muenchen *et al.*, 2000; Gasparian *et al.*,

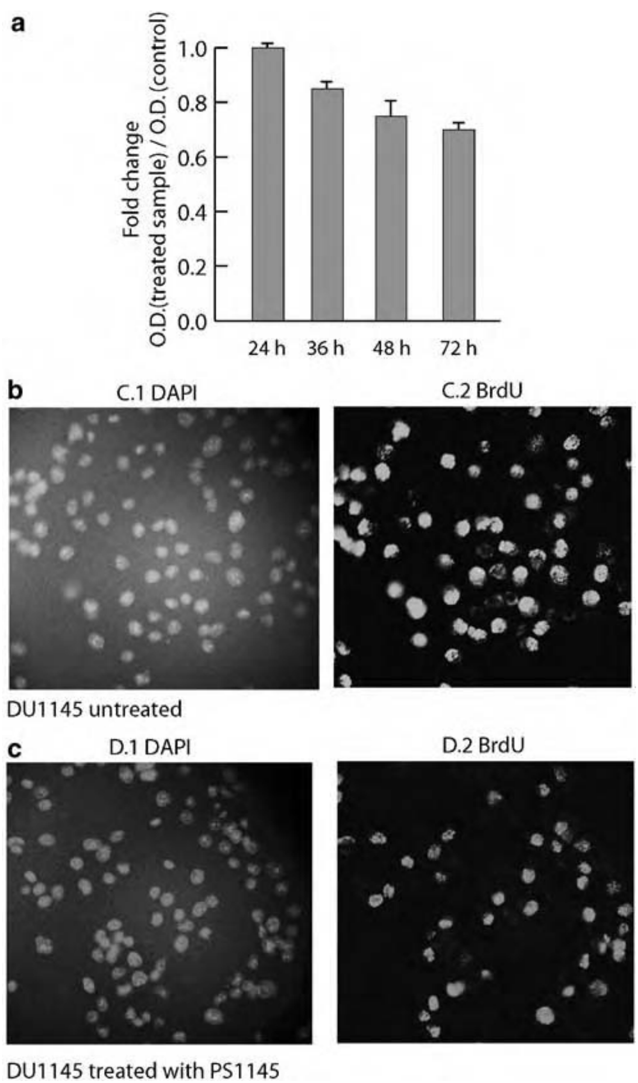

Figure 6 PS1145 inhibited proliferation of DU145 cells. (**a**) DU145 cells were treated with 10 μM PS1145 for the indicated periods of time, and cell growth was evaluated by MTS test. (**b**, **c**) Analysis of BrdU incorporation in DU1145 cells untreated (**b**) and treated with 10 mM PS1145 (**c**). Cells were treated with BrdU for 1 h, fixed in 4% formaldehyde and used for immunofluorescence with anti-BrdU antibody. DAPI nuclear staining and BrdU staining were evaluated by fluorescent microscope, $\times$320.

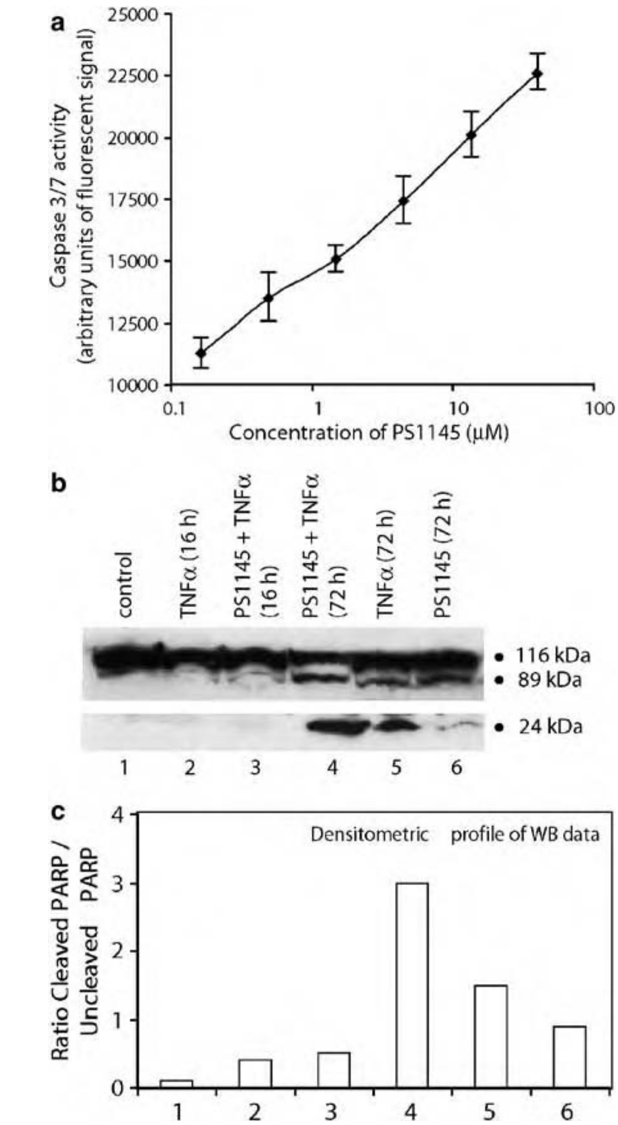

Figure 7 PS1145 induced apoptosis in DU145 cells. (**a**) Caspase 3/7 activity was evaluated in DU145 cells by ApoOne kit after cells were treated with increasing concentrations of PS1145 for 48 h. (**b**) Western Blot analysis of PARP cleavage in DU1145 cells treated with TNF-α (7 ng/ml), PS1145 (10 μM), or the combination PS1145 + TNF-α for 16 and 72 h. (**c**) Densitometric profile of Western blot analysis of PARP cleavage. The data are presented as the ratios of total cleaved PARP products of 89 and 24 kDa to uncleaved PARP.

2002; Orlowski and Baldwin, 2002; Shukla and Gupta, 2004).

Overall, our data indicate that the effect of PS1145 on PC cell growth and apoptosis develops after prolonged treatment (i.e. requires cell maintenance under the conditions when NF-κB is chronically inhibited), even though the significant NF-κB inhibition is achieved in 16 h.

PS1145 inhibited the invasion activity of PC3-S cells in vitro

It is known that DU145 cells do not possess high invasion and migration capability in *in vitro* and *in vivo* experiments. In contrast, several clones derived from the original PC3 cell line were reported to be highly invasive in animals and in invasion chambers *in vitro*. Thus, to study the effect of PS1145 on PC cell motility and invasiveness, we used a highly invasive PC3 clone PC3-S (Lindholm *et al*., 2000). The cell invasiveness was studied using invasion assay of radioactively labeled cells. As shown in Figure 8, PS1145 dramatically inhibited invasion of PC3-S cells in a dose-dependent manner. The effect was more pronounced if the cells were preincubated with PS1145 for 24 h. The inhibition of invasion by PS1145 was not due to PS1145 toxicity for PC3-S cells. PC3-S cells appeared to be rather resistant to the toxic effect of PS1145 evaluated by

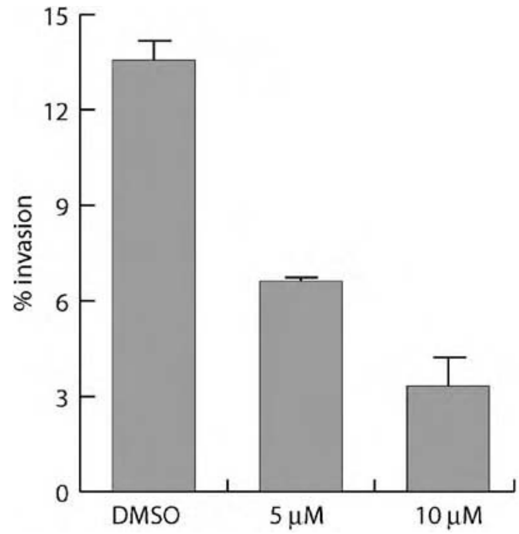

Figure 8 PS1145 inhibited the invasion capacity of PC3-S highly invasive clone. PC3-S cells were pretreated with 5 and 10 μM PS1145 for 24 h and incubated in the Transwell® invasion chamber for up to 72 h. The data are presented as percent of invasion calculated by dividing the invading cell-associated c.p.m. to the total cell-associated c.p.m. (see Materials and methods).

flow-cytometric analysis with propidium iodide (data not shown).

PS1145 effects on gene expression in PC cells

NF-κB regulates a wide variety of genes that encode antiapoptotic proteins, cell cycle proteins, cytokines, proteins involved in cell–cell and cell–extracellular matrix (ECM) interactions and others. Thus, to address the mechanisms underlying the effects of PS1145 on PC cells, we performed semiquantitative RT–PCR analysis of the set of 21 genes known to be important for control of cell cycle, apoptosis and cell–cell and cell–ECM interactions (Toth *et al*., 1995; Glasgow *et al*., 2000; Catz and Johnson, 2001; Hinz *et al*., 2001; Gupta *et al*., 2002; Martone *et al*., 2003). The gene selection was made after the comparison of database obtained by cDNA array analysis of global effect of PS1145 on gene expression in PC cells (these data are not shown in this paper, and are planned to be used for another research project) and the literature database for NF-κB-dependent genes (http://www.nf-kb.org). The list of selected genes is presented in Table 1. The quantitative analysis of gene expression was performed by Agilent 2001 Bioanalyzer as described in Materials and methods.

As shown in Figures 9 and 10, we found that the expression of nine out of 21 studied genes was significantly changed in DU145 cells treated with PS1145, especially after longer 72 h treatment. Figure 9 represents the agarose electrophoresis analysis of RT–PCR products. Figure 10 shows quantitative analysis of expression of all the selected genes (Table 1). As expected, the expression of well-known NF-κB-dependent genes such as inhibitor of apoptosis (IAP)-1, IAP-2, cyclin D1, D2, interleukin (IL)-6 and IL-9 was

Table 1 Genes studied in DU145 and PC3 cells treated with PS1145

Gene group	*Gene name*
1. Apoptosis-related genes	IAP1; IAP2; XAF1; BCL2; BAX; AVEN; c-FLIP, AVEN
2. Proliferation-related genes	Cyclin D1, Cyclin D2, Cyclin B1, Cyclin B2, Cdk4, Cdk6, Cdc2, Cdc5, Cdc6, Cdc25B
3. Angiogenesis-related genes	VEGF-A, VEGF-C, VEGF-D
4. Adhesion molecules	N-cadherin1, ICAM-1
5. Cytokines	IL-6, IL-9

significantly decreased in DU145 cells with inhibited NF-κB activity. The similar inhibition of those genes was found in PC3 cells (data not shown). The expression of three other apoptosis-related genes c-FLIP (CASP8 and FADD-like apoptosis regulator precursor, long isoform), XAF1–X-linked inhibitor of apoptosis protein (XIAP)-associated factor and cell death regulator AVEN was increased in both PC cell cultures, while we did not find any changes in expression of Bcl-2 and Bax. The relevance of those changes to apoptosis induced by PS1145 in PC cells will be discussed below. We also did not find significant changes in the expression of cyclin B1 and B2, and Cdks in PC cells, even though some of those cell cycle-related genes have been previously reported to be downregulated by NF-κB inhibitors (Guttridge *et al*., 1999; Gupta *et al*., 2002). Genes from the vascular endothelial growth factor (VEGF) family were differentially regulated in two PC cell lines: VEGF-C was significantly downregulated only in DU145 cells and VEGF-D was downregulated only in PC3 cells (Figure 10). Overall, the antiapoptotic and antiproliferative effects of PS1145 correlated well with downregulation of IAP-1, IAP-2, cyclin D1 and D2.

It is known that cytokine IL-6 plays an important role in the growth of androgen-independent prostate tumor cells via autocrine and paracrine mechanisms (Giri *et al*., 2001; Zerbini *et al*., 2003; Culig *et al*., 2004). To extend our finding on the inhibition of IL-6 gene expression by PS1145 in DU145 cells, we used ELISA assay to evaluate the amount of IL-6 protein secreted into the cell culture medium by DU145 cells treated with PS1145. As shown in Figure 11, inhibition of IKK resulted in a significant decrease of IL-6 levels.

Effect of PS1145 treatment on cell signaling pathways

The data obtained in our studies indicate that, despite the strong effect of PS1145 on NF-κB activity, its effect on proliferation and apoptosis in PC cells was more modest. This raised the question about the possible activation of some proproliferative, such as mitogen-activated protein kinase (MAPK) (Zerbini *et al*., 2003), and antiapoptotic, such as Akt (Culig *et al*., 2004; Li *et al*., 2005), signaling pathways in PC cells to compensate for NF-κB blockage. To assess the effect of PS1145 on Akt, MAPK and stress-activated protein kinase/Jun-N-terminal kinase (SAPK/JNK) signaling, we evaluated the levels of Akt, SAPK/JNK and dual-specificity mitogen-activated protein kinase kinase 1

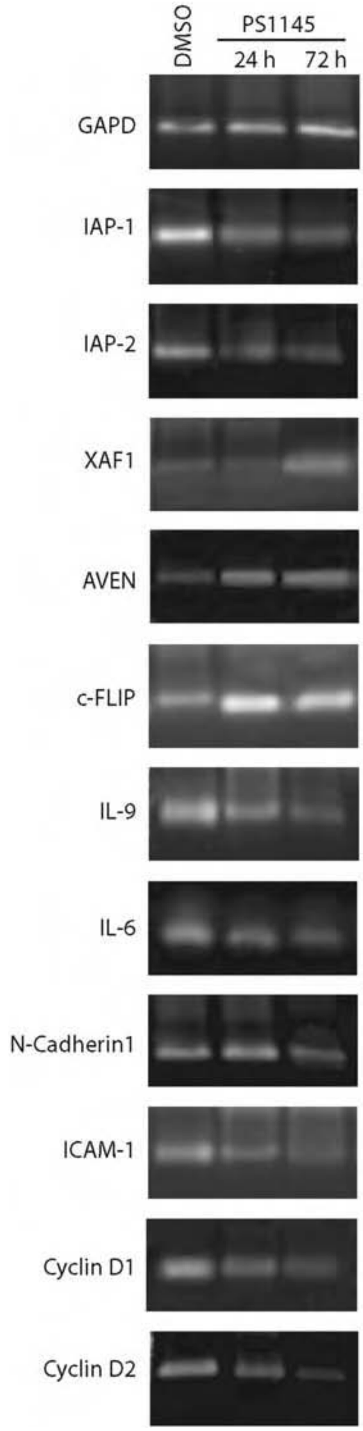

Figure 9 PS1145 effects on the expression of selected genes in DU145 cells. Agarose gel analysis of RT–PCR products of selected genes in DU145 cells treated with 10 μM PS1145 for 24 or 72 h.

(Mek1/2) phosphorylation in DU145 cells treated with PS1145 for 24 and 72 h. As shown in the Figure 12, there was no change in the phosphorylation level of SAPK/JNK kinases. Against expectations, Akt activity was inhibited by long, 72-h treatment of DU145 cells with PS1145. Interestingly, phosphorylation of c-Raf and downstream Mek1/2 was strongly, even though temporarily, increased in response to PS1145 treatment. This correlated very well with increased phosphorylation of downstream Mek1/2 target kinases extracellular signal-regulated kinase (Erk)1/2, that was mostly pronounced 24 h after treatment with PS1145.

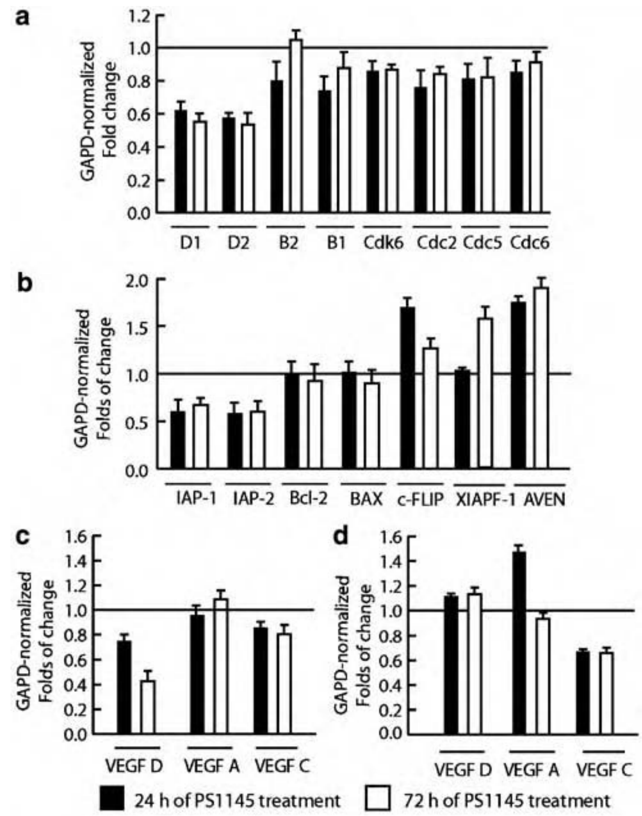

Figure 10 Quantitative analysis of PS1145 effect on gene transcription in DU145 and PC3 cells. RT–PCR products were quantitatively analysed using Agilent 2002 Bioanalyzer. The data are shown as GAPDH-normalized fold change factor calculated for each gene as GAPD-normalized amount of RT–PCR product from PS1145-treated DU145 cells divided by that of untreated DU145 sample. (**a**) Analysis of expression of cell cycle-related genes in DU145 cells. (**b**) Analysis of apoptosis-related genes in DU145 cells. (**c**) Expression of angiogenesis-related genes in PC3 cells. (**d**) Expression of angiogenesis-related genes in DU145 cells.

Discussion

There is mounting evidence that NF-κB activation is associated with tumorigenesis. NF-κB was found to be activated in human leukemias and lymphomas, lung and breast carcinomas, as well as in numerous cell lines of different origin (Rayet and Gelinas, 1999; Karin *et al*., 2002). The chronic activation of NF-κB in tumor cells has been linked both to genetic changes and to epigenetic mechanisms. There are numerous reports indicating that upstream signaling pathways causing (or associated with) tumor development can activate NF-κB. Viral oncoproteins including Tax and EVB nuclear antigen are known to activate NF-κB through

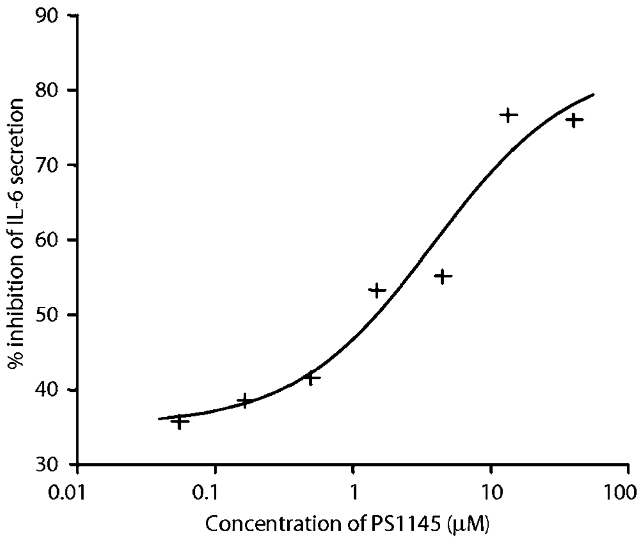

Figure 11 Analysis of PS1145 effect on IL-6 protein expression in DU145 cells. Cell culture medium was harvested 48 h after treatment of DU145 cells with PS1145 at increasing concentrations, and IL-6 expression was evaluated in cell culture medium by ELISA (Quantikine assay).

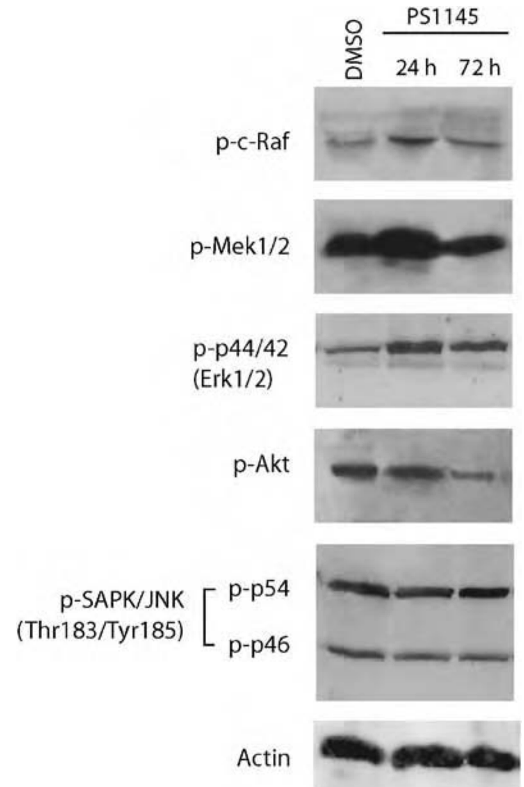

Figure 12 Analysis of PS1145 effect on cell signaling pathways. Western blot analysis of whole-cell protein extracts (50 μg/well) prepared from DU145 cells treated with 10 μM PS1145 for the indicated periods of time. Western blot membranes were probed for expression of phosphorylated c-Raf, Mek1/2, Erk1/2, SAPK/JNK, Akt and actin as a control for loading.

interaction with IKK complex or some other mechanisms (Karin *et al*., 2002; Orlowski and Baldwin, 2002). NF-κB and IKK complex could be induced by activated oncogens Ras, Bcr–Abl, members of Rho protein family (Orlowski and Baldwin, 2002). Production of numerous growth factors and cytokines that are strong activators of IKK complex, and whose expression is in turn NF-κB-dependent, is typical for tumor cells (Orlowski and Baldwin, 2002; Zerbini *et al*., 2003; Greten and Karin, 2004). Those cytokines, including IL-6 and growth factors, may contribute to the establishment of positive autocrine/paracrine loops of NF-κB activation in tumor cells (Giri *et al*., 2001; Zerbini *et al*., 2003; Culig *et al*., 2004). There is also evidence that IKK-independent pathways, including p65 phosphorylation, can be involved in NF-κB constitutive activation in tumor cells (Viatour *et al*., 2005).

Recently, we and others found that NF-κB is activated in androgen-independent PC cells and in prostate tumors, where NF-κB has nuclear localization in at least 15% of cells (Palayoor *et al*., 1999; Gasparian *et al*., 2002; Ross *et al*., 2004; Shukla *et al*., 2004; Sweeney *et al*., 2004). The major mechanism of NF-κB activation in PC cell lines involves the aberrant activation of IKK complex, resulting in increased phosphorylation and instability of IκB inhibitor proteins (Gasparian *et al*., 2002). Importantly, in this work using immunostaining with antibodies against activated, phosphorylated IKKs, we showed for the first time that IKK complex is also activated in PC samples. Thus, IKKs, especially IKKβ that is critical for NF-κB activation, represent a novel important target for NF-κB blockage in PC and other tumor cells. Very recently, several pharmaceutical companies have started working on the design of potent orally active IKKβ inhibitors (Burke *et al*., 2003; Kishore *et al*., 2003; Baxter *et al*., 2004; Murata *et al*., 2004; Ziegelbauer *et al*., 2005). PS1145 is one of these highly specific IKK inhibitors (IC < 0.1 μM) recently developed by Millenium Pharmaceuticals, Inc. (Hideshima *et al*., 2002; Lam *et al*., 2005).

In this work, we developed a comprehensive picture of the effects of PS1145 on NF-κB activity, growth, sensitivity to apoptosis and invasiveness of PC cells. We first demonstrated that pretreatment with PS1145 (10–20 μM) efficiently inhibited both basal and induced NF-κB activity in PC cells. Then we studied the major biological responses of PC cells resulting from NF-κB inhibition. We showed that PS1145 inhibited proliferation of DU145 cells when cells were incubated with IKK inhibitor for 48–72 h. These data are in line with the previous findings indicating that NF-κB is an important regulator of cell proliferation, and its effect is mediated through regulation of expression of cyclins (especially cyclin D1), possibly CDK/CKI genes, and some other cell cycle-related genes, for example, c-*myc*. Our study has revealed that PS1145 significantly inhibited cyclin D1 and D2 expression in PC cells, but did not affect the expression of cyclin B1, B2, cdk6, cdk4, cdc2, cdc5 and cdc6. Relatively modest inhibition of PC cell proliferation by PS1145 correlates well with the results obtained

in multiple myeloma cells treated with the same IKK inhibitor (Hideshima *et al*., 2002), and in PC-3 cells transfected with IκBα super-repressor (Huang *et al*., 2001), and may reflect the existence of compensatory mechanisms that counteract NF-κB blockage in PC cells. Indeed, we found that Raf/Mek1/2/Erk1/2 kinases were strongly activated in DU145 cells by PS1145. This suggests that simultaneous inhibition of NF-κB and MAP kinase cascade may result in more profound inhibition of PC cell proliferation.

A key role of NF-κB in cell protection against diverse apoptotic stimuli including chemo- and radiotherapy is very well known. The antiapoptotic NF-κB-regulated genes include genes that encode Bcl-2-like proteins (A1/Bfl1, Bcl-X_l and Nr13), IAP proteins – IAP-1, IAP-2, X-IAP-1 and others (Barkett and Gilmore, 1999). In our experiments, PS1145 itself triggered modest apoptosis in DU145 cells treated for 72 h or longer. Most importantly, PS1145 treatment significantly sensitized relatively resistant DU145 cells to TNFα-induced apoptosis. These data correlate well with the previous findings that NF-κB blockage by overexpression of nondegradable IκBα mutant may result in apoptosis or in sensitization to TNF-α-induced apoptosis in PC cells. Further analysis of mechanisms of apoptotic death induced by PS1145 in PC cells revealed the central role of caspase 3/7 in this process. Indeed, we found that PS1145 induced caspase 3/7 activation and, consequently, increased cleavage of PARP, a target protein for caspase 3/7. In turn, the activation of caspase 3/7 correlated with the decreased expression of its inhibitors, IAP-1 and IAP-2 after PS1145 treatment. Moreover, our data indicated the role of XIAP-associated factor-1 (XAF1), an antagonist of another inhibitor of caspase 3/7, X-IAP. We found that XIAF1 expression was significantly increased in PC cells treated with PS1145. On the contrary, we did not find changes in the expression of genes involved in mitochondrial apoptosis (Abraham and Shaham, 2004; Rapp *et al*., 2004). Unexpectedly, certain antiapoptotic genes have been activated in PC cells after PS1145 treatment. For example, the expression of genes that encode caspase 8 (FLICE) inhibitory protein c-FLIP (long FLIP isoform), and especially cell death regulator Aven was increased in DU145 cells treated with PS1145. Aven was recently shown to bind both Bcl-x(L) and the caspase 9 regulator Apaf-1, thus inhibiting mitochondrial apoptosis (Chau *et al*., 2000; Figueroa *et al*., 2004; Peter, 2004). Interestingly, the effect of PS1145 on Aven expression was especially pronounced in PC3 cells more resistant to PS1145-induced apoptosis, than in DU145 cells (data not shown). This may potentially explain the known higher resistance of PC3 cells to apoptosis induced by NF-κB blockage.

As we mentioned, the specific pharmacological IKK inhibitors have been developed only recently. Thus, the information about their effect on tumor cell behavior is very limited. PS1145 was recently tested in multiple myeloma cells (Hideshima *et al*., 2002). Another novel IKK inhibitor, BMS-345541 (Burke *et al*., 2003), was studied in human melanoma cells (Yasui *et al*., 2003). Overall, the effects of IKK inhibitors in other tumor cells were similar to our findings in PC cells. Both IKK inhibitors decreased tumor cell proliferation *in vitro*, and either induced apoptosis or sensitized tumor cells to apoptosis induced by TNF-α. BMS-345541 also inhibited melanoma cell growth *in vivo* as xenografts in nude mice. Interestingly, in both cell types, IKK inhibition has resulted in abrogation of paracrine growth loops, mediated in multiple myeloma cells by IL-6, and in melanoma cells by chemokine CXCL1. Aberrant expression of IL-6 has been implicated in PC progression and resistance to chemotherapy (Giri *et al*., 2001; Zerbini *et al*., 2003; Culig *et al*., 2004). IL-6 is highly expressed in androgen-independent PC cell lines, and has been shown to function as an important growth factor in PC cells, possibly also through autocrine growth loop (Zerbini *et al*., 2003). Importantly, treatment of PC cells with PS1145 resulted in significant decrease of IL-6 gene expression and decreased concentration of IL-6 protein in cell culture medium, suggesting that PS1145 affects positive growth loop mediated by IL-6 in PC cells.

In conclusion, the presented results obtained in PC cell cultures suggest that constitutively active antiapoptotic and proproliferative NF-κB signaling represents a rational target for PC treatment, especially in combination with some other proapoptotic chemotherapeutical drugs. The development of IKK inhibitors that more specifically block NF-κB signaling than all other agents including proteasome inhibitors will be very helpful to block NF-κB as a novel anticancer strategy in clinics.

Materials and methods

Cell cultures and treatments

DU145 and PC3 cells were purchased from the American Tissue Culture Collection (Rockville, MD, USA). DU145 and PC3 cells were cultured in RPMI 1640 medium (Gibco BRL Life Technologies, Rockville, MD, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA), sodium pyruvate (1 mM, Sigma Chemical Co., St Louis, MO, USA) and antibiotics (Gibco BRL Life Technologies, Rockville, MD, USA). The following reagents were used for cell treatments: PS1145 (Millenium Pharmaceuticals, Inc., Boston, MA, USA), LPS, DMSO and TNF-α from R&D Systems (Minneapolis, MN, USA). PS1145 was dissolved in DMSO, and stock solution was stored at −20°C. PC3 cells stably expressing d.n. IKKβ mutant tagged with FLAG were generated using lentivirus system from Invitrogen Corporation (Carlsbad, CA, USA) following the manufacturer's protocol. Antibiotic blasticidin was used at concentration of 6 μg/μl to select for d.n. IKKβ-expressing clones. The d.n. IKKβ expression was confirmed using anti-FLAG antibodies (Sigma Chemical Co., St Louis, MO, USA) and anti-IKKα/β antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) by Western blotting and immunostaining (data not shown). We used in our work pulled PC3-d.n. IKKβ cell cultures.

Preparation of cellular extracts and electrophoretic mobility shift assay

Nuclear and cytosolic proteins were isolated as described previously (Lyakh *et al*., 2000). The binding reaction for

EMSA contained 10 mM HEPES (pH 7.5), 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 0.5 μg of poly(dI-dC), 0.5 μg of sonicated salmon sperm DNA, γ-^{32}P-labeled (2–3 $\times$ 10^5 c.p.m.) double-stranded κB-consensus oligonucleotide (Promega Corp., Madison, WI, USA), γ-^{32}P-labeled (2–3 $\times$ 10^5 c.p.m.) and 5–10 μg of the nuclear extract. DNA-binding reaction was performed at room temperature for 30 min in a final volume of 20 μl. DNA–protein complexes were analysed on 6% DNA retardation gels (Novex, Carlsbad, CA, USA). Dried gels were subjected to radiography.

Western blot analysis

Whole-cell protein extracts were prepared using RIPA buffer as described elsewhere (Rosenberg, 1996). Proteins were resolved by electrophoresis on 10% SDS–PAGE and transferred to Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA). Anti-phospho-Mek1/2, anti-phospho-Erk1/2, anti-phospho-Akt, anti-phospho-SAPK/JNK, anti-phospho-c-Raf and anti-phospho-Ser32 IκBα and anti-PARP Abs were from Cell Signaling Technology, Inc. (Beverly, MA, USA); anti-p65 and anti-IκBα antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were blocked with 5% non-fat milk in TBST buffer and incubated with primary antibodies overnight at 4°C. Peroxidase-conjugated anti-rabbit or anti-mouse IgG (Sigma Chemical Co., St Louis, MO, USA) were used as secondary antibodies. ECL Western blotting reagent (Amersham Pharmacia Biotech, Sweden) was used for protein detection. To verify for equal amounts of proteins loaded and transferred, the membranes were stained with Ponceau Red.

Transfection of PC cells and luciferase activity

The following constructs were used for transfections: κB-luciferase reporter – *Firefly* luciferase (FL) under a 5 $\times$ κB promoter kindly provided by Dr WC Greene (Gladstone Institute for Virology and Immunology, University of California, San Francisco, CA, USA); pRL-null construct – *Renilla* luciferase (RL) under a minimal promoter (Promega Corp., San Luis Obispo, CA, USA); pcDNA3.1-CMV-IKKβ wild type (w.t.) (kindly provided by Dr F Mercurio, Signal Pharmaceutical, Inc., San Diego, CA, USA). PC cells were plated onto 12-well plates and at 50% confluency were cotransfected with indicated plasmids using Effectene reagent (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. The amount of each plasmid DNA was 0.5 μg/well. Prostate cells were harvested 36 h after the transfection and the Luciferase activity was measured by dual luciferase assay (Promega Corp., San Luis Obispo, CA, USA) on a TD20/20 Turner luminometer (Turner Design Instruments, Sunnyvale, CA, USA). When necessary, before transfections, cells were pretreated with PS1145 (10 μM) and/or TNF-α (7 ng/ml) or LPS (1.5 μg/ml). FL activity was normalized against RL activity to equalize for transfection efficacy.

Northern blot analysis

Total RNA from freshly harvested cells was isolated by TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and subjected to Northern blotting. In all, 20 μg of total RNA was resolved in 1% agarose–6% formaldehyde gel. The RNA was transferred to nylon membranes and probed for IκBα. The DNA probe was prepared by random-primed reactions using the complete coding sequence of human IκBα cDNA (ATCC, Rockville, MD, USA).

RT–PCR

Two-step RT–PCR reaction using reverse transcriptase MLV-RT with random primers and PCR-Supermix (both from Invitrogen Corp., Carlsbad, CA, USA) with appropriate PCR primers was performed using total RNA isolated by RNAeasy mini kit (Qiagen Inc., Valencia, CA, USA). PCR primers were designed using Primer-Bank database (http://pga.mgh.harvard.edu/primerbank/) and RTPrimerDB Real Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb/index.php). PCR products were analysed by electrophoresis in 1.5% agarose gel. The actual amount of PCR product was measured by an Agilent 2001 Bioanalyzer and normalized to the amount of GAPD PCR product. For quantitative analysis, the data are represented as the ratio of GAPD-normalized amount of PCR product in PS1145-treated cells to the GAPD-normalized amount of PCR product in control cells.

IL-6 secretion

DU145 cells were plated at 5000 cells/well in BD356640 poly-D lysine 96-well plates (Beckton Dickenson, Franklin Lakes, NJ, USA). Cells were incubated with PS1145 at increasing concentrations for 48 h. IL-6 level in tissue culture media was measured using Quantikine Assay for human IL-6 (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol using Wallace Victor 2 1420 luminometer.

Assessment of proliferation

Cell proliferation was assessed using None-Radioactive Cell Proliferation Assay (MTS test) from Promega Corporation (San Luis Obispo, CA, USA) and bromodeoxyuridine (BrdU) cell labeling using immunofluorescence. For both tests PC were plated onto 12-well plates (20 $\times$ 10^3 cells/well), and cultured for 24–72 h in the presence of 10 μM PS1145. Every 24 h fresh complete media containing 10 μM PS1145 was added to the cells. For MTS test each group of cells was plated in triplicate. The MTS reagent was prepared and used according to the manufacturer's protocol. Optical density of the samples was measured on a plate reader at 490 nm.

For BrdU labeling, the cells were plated onto sterilized coverslips placed on the well bottoms in 12-well plates. PS1145-pretreated and control DU145 cell cultures were treated with BrdU (2 μg/ml) for 2 h. The coverslips with cells were fixed and permeabilized with acetone–methanol (1:1) mixture at $-20°$ for 15 min, washed with PBS, blocked with 20% goat serum and stained with primary anti-BrdU Ab from Becton Dickinson (Franklin Lakes, NJ, USA) and secondary goat anti-mouse Ab conjugated with Cy3 (Jackson Immunoresearch laboratories Inc., West Grove, PA, USA). DAPI (Vector laboratories, Burlingame, CA, USA) was used to counterstain the nuclei. The number of BrdU-positive cells was counted in PS1145-treated and control cultures (10 fields of view in each sample). For quantitative analysis, the data were presented as the % of BrdU-positive nuclei to the total number of nuclei stained with DAPI.

Apoptosis detection

To evaluate apoptosis, we used Western blot analysis of PARP proteolysis and caspase 3/7 functional assay. To study PARP proteolysys, prostate cells, plated on 10 mm dishes, were treated with 10 μM PS1145 alone or in combination with 7.5 ng/ml TNF-α (R&D Systems, Minneapolis, MN, USA) for 16–72 h upon reaching 50% confluence. Adherent cells and detached floaters were combined for whole-cell protein extract preparations. PARP cleavage was estimated by Western blot analysis with anti-PARP antibody (Pharmingen, San Diego, CA, USA).

For caspase 3/7 functional assay, DU145 cells were plated at 5×10^3 cells/well of a 24-well plate in 100 μl of complete media. PS1145 was added at increasing concentrations from 0.1 to 50 μM. Caspase activity was measured after 48 h treatment with PS1145 using ApoOne kit (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. In all, 100 μl of the substrate was added for 2.5 h.

Transwell® invasion assays
The PC-3 High Invasion subclone (PC3-S) was previously selected by serial passages through reconstituted basement membrane Matrigel® (Becton Dickinson, Lincoln Park, NJ, USA) in the Transwell® invasion apparatus (Lindholm *et al*., 2000). For analysis of PC-3S cell invasion in the presence of PS1145 inhibitor, a Transwell® invasion assay was used according to the protocol described previously (Lindholm *et al*., 2000). Prior to the invasion assay, the cells were preincubated with [^{3}H]thymidine and either PS1145 or control vehicle (Lindholm *et al*., 2000) overnight. The cells were incubated in the invasion chamber for up to 72 h. Invading cells were collected as described previously (Lindholm *et al*., 2000), and the cell invasion was quantitated by counting the cell-associated c.p.m. The percent invasion was calculated by dividing the invading cell-associated c.p.m. to the total cell-associated c.p.m. The statistical analysis of percent invasion was determined by Student's *t*-test comparisons using InStat™ statistical software (GraphPad Software, Inc., San Diego, CA, USA). The results are presented as mean ± standard deviation (s.d.).

Immunostaining of prostate tissues
Prostate tissues were obtained from white male patients at the age 40–82 years during biopsy or surgery to remove prostate tumors. Paraffin sections of formalin-fixed PC samples with verified diagnosis were used for immunostaining. The immunostaining was performed using Envision + System-HRP (DAB) kit according to the manufacturer's protocol (DakoCytomation, Carpenteria, CA, USA). After Ag retrieval in a pressure cooker (for 5 min at 20–25 psi) in citric buffer (pH 6.0), tissue sections were blocked with 20% goat serum in PBS, and consequently incubated with primary rabbit polyclonal Ab against phospho-IKKα/β (Cell Signaling Technology, Inc., Beverly, MA, USA), followed by secondary anti-rabbit IgG-reagent provided with the Envision + System-HRP (DAB) kit. Immunostaining was visualized with DAB chromogen (Dako-Cytomation, Carpenteria, CA, USA) and counterstained in Mayer's hematoxylin.

Data in all figures are shown as results of the representative experiments. All experiments were repeated at least three times.

Abbreviations

EMSA, electrophoretic mobility shift assay; Erk, extracellular signal-regulated kinase; IAP, inhibitor of apoptosis; IKK, inhibitor of nuclear factor kappa-B kinase; IκB, inhibitor of nuclear factor kappa-B; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Mek1/2, dual-specificity mitogen-activated protein kinase kinase 1; NF-κB, nuclear factor kappa-B; PC, prostate carcinoma; SAPK/JNK, stress-activated protein kinase/Jun-N-terminal kinase; TNF-α, tumor necrosis factor alpha.

Acknowledgements
We thank Dr WC Greene (Gladstone Institute for Virology and Immunology, University of California, San Francisco, CA, USA) and Dr F Mercurio (Signal Pharmaceutical, Inc., San Diego, CA, USA) for their kind gift of plasmids. This work was supported by DOD prostate grants DAMD17-01-1-0015 and DAMD17-03-1-0522 (to IB), Northwestern University Prostate SPORE Developmental Project (to IB), and Russian Foundation for Basic Research, grant # 0404-49240a (to AG).

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Oncogene (2006) 00, 1–12

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ORIGINAL ARTICLE

Tumor suppressor activity of glucocorticoid receptor in the prostate

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Glucocorticoids are extensively used in combination chemotherapy of advanced prostate cancer (PC). Little is known, however, about the status of the glucocorticoid receptor (GR) in PC. We evaluated over 200 prostate samples and determined that GR expression was strongly decreased or absent in 70–85% of PC. Similar to PC tumors, some PC cell lines, including LNCaP, also lack GR. To understand the role of GR, we reconstituted its expression in LNCaP cells using lentiviral approach. Treatment of LNCaP-GR cells with the glucocorticoids strongly inhibited proliferation in the monolayer cultures and blocked anchorage-independent growth. This was accompanied by the upregulation of p21 and p27, downregulation of cyclin D1 expression and c-Myc phosphorylation. Importantly, the activation of GR resulted in normalized expression of PC markers hepsin, alpha-methylacyl-CoA racemase (AMACR) and maspin. On the signaling level, GR decreased expression and inhibited activity of the mitogen-activated protein kinases (MAPKs), including p38, c-Jun NH_2-terminal kinase/stress-activated protein kinase, dual-specificity mitogen-activated protein kinase 1 and 2 and extracellular signal-regulated kinase 1 and 2. We also found that activation of GR inhibited activity of numerous transcription factors (TF), including activator protein 1, serum-responsive factor, nuclear factor kappa-B, p53, activating transcription factor 2, CEBPα, Ets-1, Elk-1, signal transducer and activator of transcription 1 and others, many of which are regulated via MAPK cascade. The structural analysis of hepsin and AMACR promoters provided the mechanistic rationale for PC marker downregulation by glucocorticoids via inhibition of specific TFs. Our data suggest that GR functions as a tumor suppressor in prostate, and inhibits multiple signaling pathways and transcriptional factors involved in proliferation and transformation.
Oncogene (2006) **0,** 000–000. doi:10.1038/sj.onc.1209991

Keywords: prostate carcinoma; PIN; glucocorticoid receptor; PC marker; transcription factor; MAPKs

Introduction

Glucocorticoid hormones regulate proliferative, inflammatory and immune responses. For years, glucocorticoids have been extensively used for the treatment of hormone refractory prostate cancer (HRPC), and the combination of paclitaxel and dexamethasone remains a standard treatment for HRPC patients in the US and other countries (reviewed by Fakih *et al*., 2002). Glucocorticoids have also been used as the 'standard' therapy arm in several randomized phase II–III clinical trials for the combination therapy of HRPC (Fakih *et al*., 2002; Koutsilieris *et al*., 2002).

The cellular response to glucocorticoids is mediated through a highly specific glucocorticoid receptor (GR). In the absence of glucocorticoids, GR is sequestered in the cytoplasm by chaperone proteins. Following ligand binding, the GR dissociates from the chaperones and forms homodimers, which enter the nucleus. There are two major mechanisms of gene regulation by GR (De Bosscher *et al*., 2003; Necela and Cidlowski, 2004). The Q1 direct positive transcriptional regulation (transactivation) occurs via binding of the GR homodimer to palindromic promoter DNA sequences called glucocorticoid-response elements. The indirect regulation is mediated via crosstalk with other transcription factors (TFs), including activator protein 1 (AP-1), nuclear factor kappa-B (NF-κB), signal transducer and activator of transcription (STAT)-5, SMAD3, etc. (De Q2 Bosscher *et al*., 2003; Necela and Cidlowski, 2004). Q3 Most of such GR–TF interactions repress the activity of partner TFs and their target genes (transrepression). Recently, the additional mechanism of indirect gene regulation by GR has been discovered where GR blocks mitogen-activated protein kinases (MAPKs) (Kassel *et al*., 2001; Bruna *et al*., 2003). Indirect, DNA-independent mechanisms of GR gene regulation appear to be critical for the anti-inflammatory effects (Schacke *et al*., 2002), whereas their role in the growth inhibition Q4 by glucocorticoids has never been addressed.

Although the clinical effect of glucocorticoids in HRCP patients is well known, the objective responses have been found only in 20–25% of patients (Fakih *et al*., 2002). The limited effect of glucocorticoids in prostate carcinoma (PC) patients implies the changes in GR expression, function and/or availability of GR targets in PC cells. Indeed, we and others showed that different types of tumor cells lose their sensitivity to

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Received 16 June 2006; revised 27 July 2006; accepted 28 July 2006

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Journal: ONC Disk used Despatch Date: 2/9/2006
Article : npg_onc_2006-01001
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growth inhibition and apoptosis by glucocorticoids either because of the loss of GR expression or because of the abnormal GR function (Ray, 1996; Budunova *et al*., 1997; Greenstein *et al*., 2002). These observations suggest that intact GR signaling is crucial for the growth control of lymphoid and epithelial cells and that in some tissues GR may act as a tumor suppressor.

Despite the use of glucocorticoids in the standard combinational therapies of PC patients, the information regarding GR expression in PCs is surprisingly limited and conflicting (Mohler *et al*., 1996; Nishimura *et al*., 2001). To our knowledge, GR expression in early prostate lesions such as intraepithelial neoplasia (PIN) has never been evaluated. Furthermore, GR function in the prostate cells and its role in PC have never been studied, even though the growth inhibitory effect of glucocorticoids in GR-positive human and rat prostate cells has been reported (Nishimura *et al*., 2001). These previous studies chiefly attribute growth inhibitory effect of glucocorticoids to the inhibition of NF-κB TF (Nishimura *et al*., 2001).

Here, we for the first time present the comprehensive analysis of GR expression changes in the course of prostate tumorigenesis, and determine the effect of activated GR signaling on proliferation and the maintenance of transformed phenotype by PC cells.

Results

The expression of GR is decreased in HGPIN and PCs

We analysed GR expression in prostatic tissue specimens retrieved from the two independent repositories. Overall, we evaluated GR expression in 35 high-grade prostatic intraepithelial neoplasia (HGPIN) lesions, 116 PC samples (sum Gleason grades 6–10) and in 67 benign prostatic hyperplasia (BPH) samples.

The results of GR immunostaining appeared to be very similar between the cohorts (Figure 1 and Table 1). More than 80% of BPH samples showed high-intensity GR staining with nuclear localization in the epithelial cells (Figure 1a). Strong GR staining was localized to the nuclei in most of the glands in apparently normal prostatic tissues (Figure 1b). The nuclear localization strongly suggests that GR is constitutively active in both normal and hyperplastic prostate glands. In contrast, GR levels were low or below detection limit in 70–85% of PCs. There was no association between GR expression levels and Gleason grade of PCs in both cohorts. The lack of dynamics in GR expression during PC progression suggests that it is lost early in prostate tumorigenesis. Indeed, we found that GR expression was significantly decreased in 37% and partially decreased in 40–50% of HGPIN lesions compared to the morphologically normal prostate and BPH glands. However, the average number of GR-positive cells in HGPIN epithelium was almost twofold higher than in PC samples (Figure 1c). Although the analysis of prostate stroma was beyond the scope of this study, we noted that GR was present at high level in the nuclei of stromal cells (Figure 1d). Overall the immunostaining showed the decrease in GR expression to be an early event in prostate tumorigenesis, and suggested that GR may be important to control the growth of prostate cells.

Table 1 GR expression is strongly decreased in prostate carcinomas.

Tissue samples	*Patient cohorts*	*Number of samples*	*Staining intensity*		
			+/−%	++%	+++%
BPH	I	15	0	20	80
	II	52	0	4	96
HGPIN	I	30	37	53	10
	II	5	0	40	60
PC (Gl. 6–7)	I	41	68	22	10
	II	17	88	12	0
PC (Gl. 8–10)	I	30	70	20	10
	II	28	85	15	0

Abbreviations: BPH, benign prostatic hyperplasia; Gl, gleason score; GR, glucocorticoid receptor; HGPIN, high-grade prostatic intraepithelial neoplasia; PC, prostate carcinoma. GR immunostaining was analysed in two cohorts of patients from Northwestern University (I) and Russian Cancer Research Center (II). Number of epithelial cells with nuclear GR localization was evaluated by +/− to +++ scoring as described in Materials and methods.

Generation of GR-expressing LNCaP cells

To study the effect of GR re-expression on PC cell growth and transformation, we generated LNCaP cells stably expressing GR cDNA tagged with V5-tag at C-terminus using the lentiviral system. For tracking, we co-infected LNCaP-GR cells with lentivirus-expressing yellow fluorescent protein (YFP) (Figure 2a). LNCaP cells infected with the empty vector (LNCaP-V) or with the YFP-expressing lentivirus (LNCaP-YFP) were used as a negative control. The level of GR in LNCaP-GR cells was comparable to the level of endogenous GR in DU145 and PC3 prostate cells (data not shown).

In the non-stimulated LNCaP-GR cells, GR was expressed mostly in cytoplasm and in some cells in the nuclei (Figure 2b). This result probably reflects the altered ratio between GR and chaperone proteins in these cells, allowing partial spontaneous translocation of overexpressed GR in response to glucocorticoids in the serum. Upon stimulation, with fluocinolone acetonide (FA), exogenous GR readily translocated into the nuclei in ~90% cells (Figure 2c and d). As expected, the treatment of LNCaP-GR cells with FA activated the glucocorticoid-responsive TAT3.Luciferase and MMTV.Luciferase reporters (Figure 2e).

GR signaling blocked proliferation and anchorage-independent growth but did not induce apoptosis in LNCaP-GR cells

We then studied the effect of restored GR signaling on LNCaP growth in monolayer and in soft agar. We took advantage of YFP expression in the LNCaP-GR-YFP

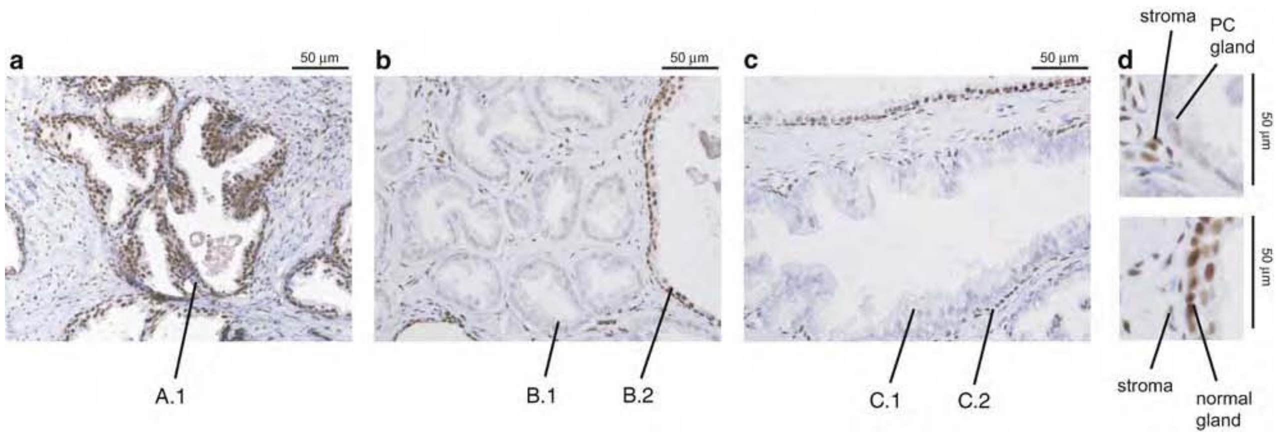

Figure 1 Expression of GR in BPH, PC and HGPIN. Immunolocalization of GR in paraffin sections of prostate tissues. (**a**) BPH; (**b**) PC (Gleason score 7); (**c**) HGPIN and (**d**) prostate stroma. Note: Low GR expression in PC (B1) and high GR expression in apparently normal prostate (B2) combined with positive nuclear GR staining in prostate stromal cells (**d**).

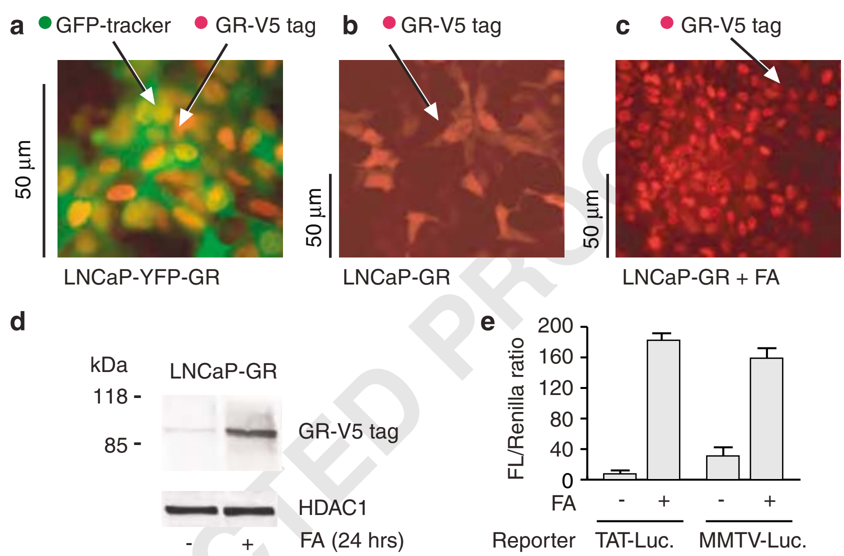

Figure 2 Characterization of GR in LNCaP-GR cells. (**a**) Monitoring of live LNCaP-GR cells. LNCaP-GR-V5 cells were co-infected with YFP-expressing lentivirus and stained with Ab against V5. (**b–d**) Glucocorticoid-induced nuclear translocation of GR. LNCaP-GR-V5 cells treated with vehicle (**b**) or FA, 10^{-7} M $\times$ 24 h (**c**) were stained with Ab against V5. (**d**) Western blot analysis of GR in nuclear protein extracts from control and FA-treated LNCaP-GR cells. (**e**) GR activity in dual Luciferase assay. FA-treated (10^{-7} M $\times$ 24 h) LNCaP-GR cells were transiently transfected with TAT- and MMTV-FL reporters, and RL reference reporter. FL activity was normalized against RL activity to equalize for transfection efficacy. The results of one representative experiment (three wells/experimental group) are shown as mean $\pm$ s.d.

cells to measure the actual number of cells/well. Glucocorticoid treatment of LNCaP-GR-YFP cells resulted in a strong growth inhibition (Figure 3a), whereas producing no significant effect on control LNCaP-V and LNCaP-YFP cells (data not shown).

On molecular level, the decreased proliferation was accompanied by upregulation of cyclin-dependent kinase inhibitors p21 and p27, decreased expression of cyclin D1 and proliferation marker Ki67, and a lower c-Myc phosphorylation (Figure 3c and d). Interestingly, the expression of p21 was increased in LNCaP-GR cells in comparison to LNCaP-V cells even without hormone treatment. This may be due to GR partial spontaneous nuclear translocation described above.

To assess the transformation levels *in vitro*, we measured anchorage-independent growth in soft agar. Even without FA, both the number and the size of the colonies formed by LNCaP-GR-YFP cells were decreased compared to the LNCaP-YFP control (data not shown). Upon glucocorticoid treatment, colony formation by LNCaP-GR-YFP cells was drastically decreased (Figure 3b).

As epidermal growth factor (EGF) signaling is important for PC growth and transition to the HRPC stage and triggers PC cell proliferation *in vitro* (Mimeault *et al*., 2003), we chose EGF as physiologically relevant stimulus to assess the GR effect on the induced PC cell growth. Although recombinant EGF signifi-

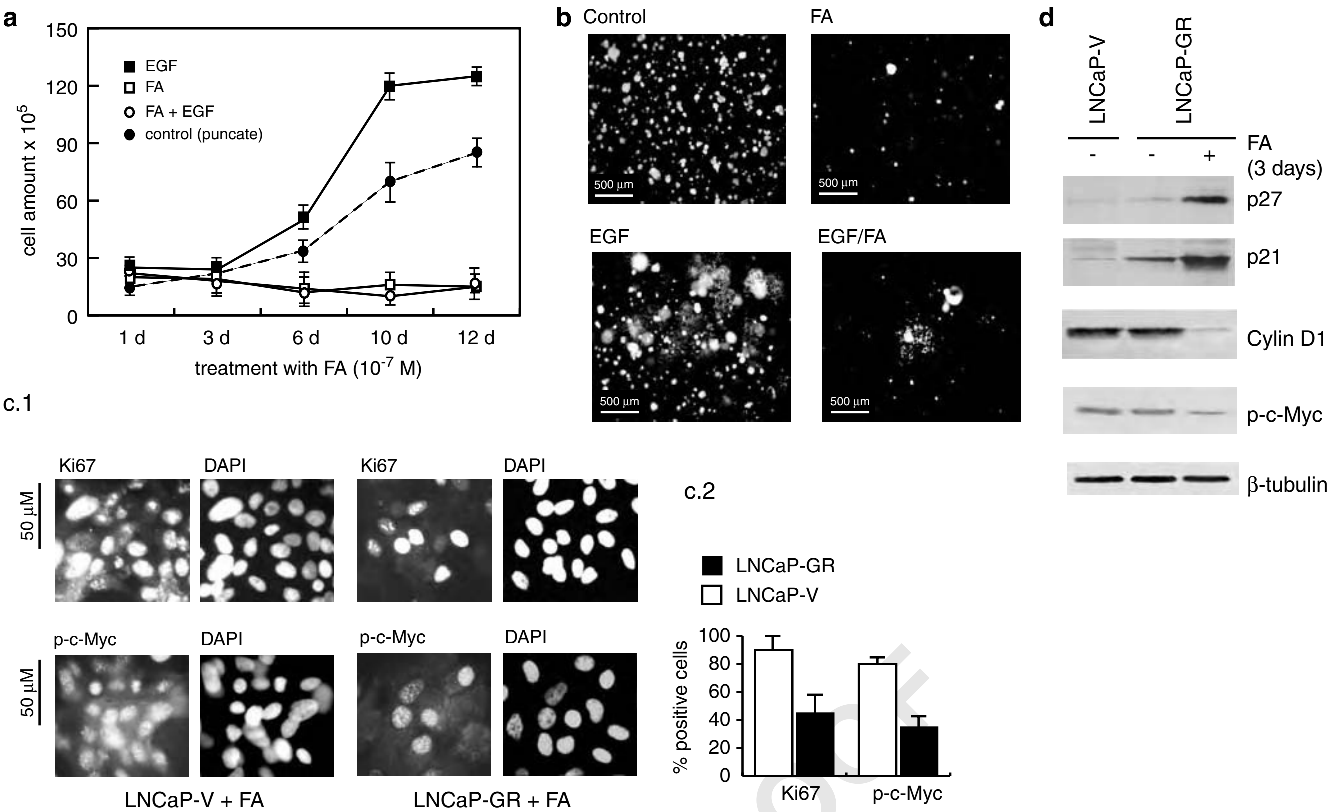

Figure 3 Inhibition of proliferation and anchorage-independent growth of LNCaP-GR-YFP cells by glucocorticoid. (**a**) Effect of FA on LNCaP-GR-YFP cell growth in monolayer. Number of LNCaP-GR-YFP cells treated with vehicle, FA (10^{-7} M), EGF (5 ng/ml) or EGF + FA was measured by YFP fluorescence using a plate reader. The results of one representative experiment (three wells/experimental group) are shown as mean ± s.d. (**b**) Effect of FA on anchorage-independent growth of LNCaP-GR-YFP cells. LNCaP-GR-YFP cells were grown in 0.6% soft agar for 2 weeks in the presence of FA (10^{-7} M), EGF (5 ng/ml), EGF + FA or vehicle (0.1% ethanol). (**c**) Immunocytochemical analysis of Ki67 proliferation marker and phosphorylated form of c-Myc. LNCaP-V and LNCaP-GR cells were treated with 10^{-7} M FA for 72 h. (c1) The immunocytochemistry results were quantitated as a percent of the positively stained cells to all cells (DAPI) in the field of view of microscope. Totally 500 cells were evaluated in each group. (**d**) Western blot analysis of cell cycle-related proteins. The expression of cyclin D1, p21, p27 and phosphorylation of c-Myc was evaluated by Western blotting in whole-cell protein extracts from LNCaP-YFP and LNCaP-GR-YFP cells treated with vehicle (−) and 10^{-7} M FA × 72 h (+).

cantly augmented the growth of LNCaP-V and LNCaP-YFP cells (data not shown) as well as LNCaP-GR-YFP cells, both in monolayer and in soft agar (Figure 3a and b), this effect was strongly inhibited by FA in LNCaP-GR-YFP cells (Figure 3a and b), but not in control cells (data not shown). Thus, activated GR strongly suppressed proliferation and anchorage-independent growth. This inhibitory effect was not attenuated by EGF, a well-known mitogen implicated in the progression of PC.

In some cell types including lymphocytes, glucocorticoid treatment may cause apoptosis (Bourcier *et al*., 2000; Greenstein *et al*., 2002). As shown in Figure 3a, FA significantly reduced the number of LNCaP cells on days 6–12 of the treatment. However, the analysis of the poly-(ADP-ribose) polypeptide (PARP) cleavage, mitochondrial potential and caspase activity in LNCaP-GR cells treated with FA have not revealed significant proapoptotic effect of glucocorticoids in PC cells (data not shown).

GR activation normalized the expression of PC markers

To further evaluate the effect of GR signaling, we investigated several early and medium/late PC markers whose expression typically changes during prostate tumorigenesis. For the profiling, we selected maspin that is usually downregulated in PCs, hepsin, which is upregulated in PCs and alpha-methylacyl-CoA racemase (AMACR) whose expression increases early, in both HGPIN and PC lesions (Chen *et al*., 2003; Ananthanarayanan *et al*., 2005). Western blot analysis and semiquantitative reverse transcriptase–polymerase chain reaction (RT–PCR) showed that in LNCaP-GR cells hepsin and AMACR were downregulated, whereas tumor suppressor maspin was upregulated upon FA treatment (Figure 4). Interestingly, the expression of PC marker genes was partially normalized in LNCaP-GR cells even without FA treatment. This could be attributed to the partial nuclear localization of GR in the untreated LNCaP-GR cells (see Figure 2). In summary, we conclude that the restoration of GR signaling resulted in overall normalization of PC cell phenotype.

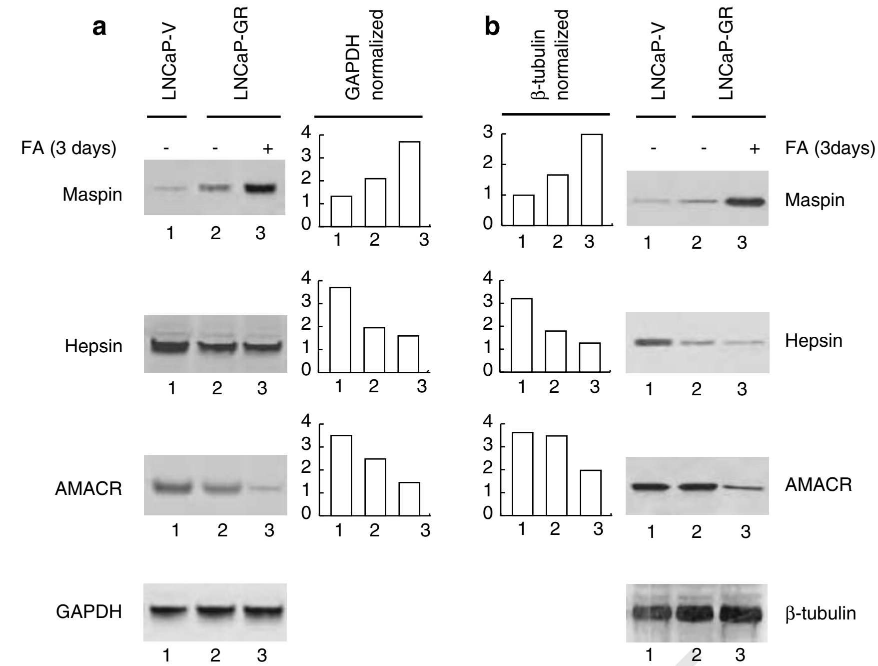

Figure 4 Effect of glucocorticoid FA on the expression of PC markers in LNCaP-GR cells. (**a**) Semiquantitative RT–PCR analysis of the PC markers expression. Total RNA from LNCaP-V and LNCaP-GR cells treated with vehicle (control) or with 10^{-7} M FA for 72 h was subjected to two-step RT–PCR. The amount of PCR products was measured and normalized to the amount of GAPDH PCR product. Quantitative data are presented as the ratio of GAPDH-normalized amount of PCR product in FA-treated vs vehicle-treated cells. (**b**) Western blot analysis of the PC markers expression. Protein expression was analysed in whole-cell protein extracts from LNCaP-V and LNCaP-GR cells treated with 10^{-7} M FA for 72. Signals were quantified as described in Materials and methods.

GR activation blocked MAPK activity in LNCaP cells

The inhibition of MAPKs is an important regulatory mechanism by GR (Kassel *et al*., 2001; Bruna *et al*., Q5 2003; Necela and Cidlowski, 2004). Therefore, we examined the GR effect on the basal and inducible activity of MAPKs dual-specificity mitogen-activated protein kinase 1 and 2 (Mek1/2), extracellular signal-regulated kinase 1 and 2 (Erk1/2), c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 using Western blot analysis with antibodies (Abs) specific for the active, phosphorylated forms of the respective kinases.

The levels of MAPK expression and phosphorylation were not affected by glucocorticoid FA in vector transfected LNCaP cells resistant to the growth-inhibitory effect of glucocorticoids (data not shown). In contrast, the LNCaP-GR cells had much lower basal levels of phospho-Mek1/2, p38 and JNK/SAPK (Figure 5a), again likely reflecting partial GR activation discussed above. FA treatment caused dramatic time-dependent decrease of MAPK phosphorylation in LNCaP-GR cells. The level of phospho-MAPKs phosphorylation was decreased by the second day of treatment and further diminished during 3–6 day course of FA treatment (Figure 5a). The relatively slow inhibition of MAPK phosphorylation by glucocorticoids is in line with the previous findings (Kassel *et al*., 2001; Greenberg *et al*., 2002).

Interestingly, our experiments revealed that glucocorticoids also reduced the total amount of MAPK proteins. Mek1/2, p38 and SAPK/JNK protein levels decreased after 24 h FA treatment, and remained at this level thereafter (Figure 5a). Semiquantitative RT–PCR analysis of Mek1, Mek2, Erk1, Erk2, p38 and JNK/SAPK has not revealed significant inhibition at the mRNA level (data not shown). Therefore, glucocorticoid treatment may have affected either translation or stability of MAPK proteins.

MAPK cascade is activated by growth factors including EGF, cytokines and stress (reviewed by Maroni *et al*., 2004). We investigated possible GR effect on induced MAPK phosphorylation using the inducers of specific MAPKs (Maroni *et al*., 2004). We used EGF for Mek1/2 and Erk1/2 activation, and interleukin (IL)-1 or tumor necrosis factorα (TNF)α for JNK/SAPK and p38 activation. IL-1 and EGF activated the corresponding MAPKs in LNCaP-V control cells within 5–15 min of treatment (Figure 5b and data not shown). In LNCaP-GR cells, the effect of studied inducers on Mek1/2 and SAPK/JNK was preserved, but the effect on Erk1/2 and p38 phosphorylation was either weak or absent even without hormone treatment (Figure 5b). FA pretreatment decreased the effects of EGF and IL-1 on MAPK activation even further (Figure 5b). Similarly, p38 and SAPK/JNK phosphorylation upon TNFα treatment was also inhibited in LNCaP-GR cells pretreated with glucocorticoids (data not shown).

The negative effect of glucocorticoids on Erk1/2 and p38 phosphorylation is known to be associated with increased expression of MAPK phosphatase 1 (MKP1)

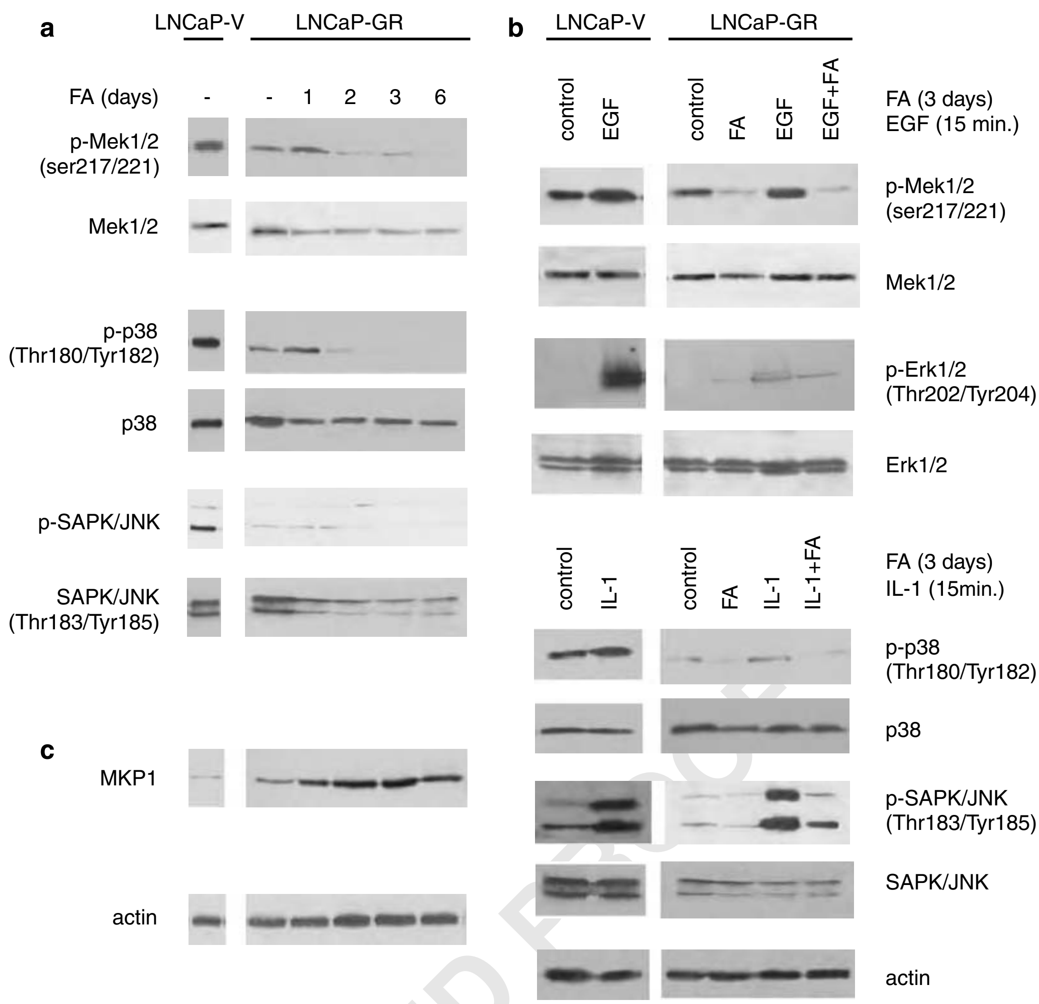

Figure 5 Inhibition of MAPK expression and activity by glucocorticoid in LNCaP-GR cells. MAPK expression and phosphorylation were analysed by Western blotting in whole-cell proteins extracts isolated from LNCaP-GR and LNCaP-V cells treated as indicated below. (**a**) Effect of FA on the constitutive MAPKs expression and activity. LNCaP-GR cells were treated with vehicle or 10^{-7} M FA for indicated time. (**b**) Effect of FA on the inducible MAPKs activity. LNCaP-GR and LNCaP-V cells were pretreated with 10^{-7} M FA for 3 days and MAPKs activity was induced by 15 min treatment with EGF (100 ng/ml); IL-1 (10 nM) or TNFα (10 ng/μl). (**c**) Effect of FA on MKP1. LNCaP-GR cells were treated with vehicle or 10^{-7} M FA for indicated time.

(Kassel *et al*., 2001). We found that the expression of MKP1 protein was indeed increased in LNCaP-GR cells treated with FA (Figure 5c).

Overall our studies strongly suggest that GR tightly regulates both constitutive and inducible activity of multiple MAPKs in PC cells.

GR-regulated TFs in PC cells

One important mechanism of gene regulation by GR involves its interaction with other TFs. We utilized novel protein–DNA array technology for simultaneous assessment of the DNA-binding activity of multiple TFs. The effect of GR on TFs was evaluated in LNCaP-GR cells after 3 days of FA treatment, the time point when MAPKs were strongly inhibited, but the effect on cell growth was modest. Vehicle-treated LNCaP-V cells were used as an additional control to evaluate constitutive DNA binding. Only reproducible $\geqslant$2-fold changes in DNA binding were pursued. We identified multiple TFs affected by activated GR in LNCaP cells (Figure 6a). Interestingly, ~85% of those TFs were downregulated. GR-activated interferon γ activation site recognized by STAT3, and orphan nuclear receptor α. Conversely, GR inhibited several TFs known to interact directly with GR and involved in transrepression such as AP-1, NF-κB, C/enhancer binding protein (C/EBP)α, activating transcription factor (ATF)-2 (CREB-BP1), p53 and SMAD3 (see references in Figure 6a). GR also blocked several TFs recognized as MAPK substrates/targets such as AP-1, SRF, Ets-1, Elk-1, STAT1, C/EBPα, GATA4, ATF-2, nuclear factor of activated T cell (NFAT)-c, PAX6 and EGR1 (see references in Figure 6a of Supplementary material #1).

To validate the results of promoter array and to investigate the functional consequences of GR-induced changes in TF-DNA binding, we employed reporter assays. Reporter constructs for NF-κB, Ets-1, Elk-1,

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a

TF name	1	2	3	Ref
Down-Regulated TF				
FRA-1/JUN AP-1 SRF	5.2 3.5 2.5	+ + +	SAPK/JNK Erk1/2	De Bosscher *et al.*, 2003; Adcock, 2001; Necela and Cidlowski, 2004; Pulvere *et al.*, 1991; Derijard *et al.*, 1994; Inostroza *et al.*, 2005; Karaganni *et al.*,1994
Ets-1 Elk-1	6.2 7.5	n/a n/a	Erk1/2, p38 SAPK/JNK	Bebied *et al.*, 2003; Foulds *et al.*, 2004; Ricote *et al.*, 2006; Koul *et al.*, 2004
STAT1/ISRE	3.1	+	Erk1/2	De Bosscher *et al.*, 2003; Adcock, 2001; Necela and Cidlowski, 2004;, Xuan *et al.*, 2005
C/EBPα	2.6	+	Erk1/2	Rudiger *et al.*, 2002; Prusty *et al.*, 2002
GATA4	3.7	n/a	Erk1/2	Liang *et al.*, 2001
ATF2	3.6	+	Erk1/2, p38, JNK1/2	van Dam *et al.*, 1995; Morton *et al.*, 2004; Almlof *et al.*, 1998
SMAD3	2.2	+	p38	Adcock, 2001; Song *et al.*,1999; Hayes *et al.*, 2003
NF-κB	3.6	+	multiple	De Bosscher *et al.*, 2003; Schacke *et al.*, 2002; Adcock, 2001; Necela and Cidlowski, 2004
p53	3.7	+	n/a	Sengupta and Wasylyk, 2001
EGR1	2.8	n/a	Erk1/2 SAPK/JNK	Rolli-Derikinderen *et al.*, 2003
PAX6	3.4	n/a	Erk1/2, p38	Mikkola *et al.*, 1999
NFAT-c	2.8	+	Erk1/2 SAPK/JNK	Adcock, 2001; Porter *et al.*, 2000; Lerner *et al.*, 2003
Up-Regulated TF				
STAT3/GAS	2.3	+	Erk1/2 SAPK/JNK	De Bosscher *et al.*, 2003; Adcock, 2001; Necela and Cidlowski, 2004; Lerner *et al.*, 2003
RORα	3.1	n/a	n/a	

b

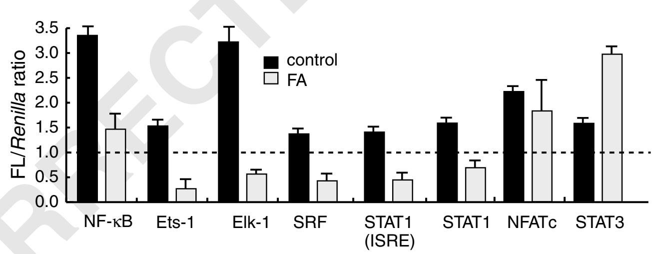
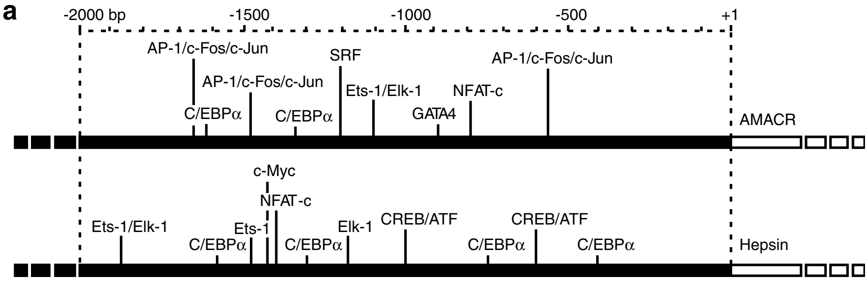


Figure 6 Analysis of glucocorticoid effect on TF activity in LNCaP-GR cells. (**a**) Analysis of multiple TF basal activity using Protein-DNA array. Nuclear protein extracts from vehicle-treated and FA-treated (10^{-7} M FA × 72 h) LNCaP-GR cells were used to analyse activity of multiple TFs (Protein-DNA interaction array, Panomics Inc.). (1) TF-DNA-binding change (folds of decrease or increase of DNA binding in FA-treated cells compared to control); (2) TF is a known partner for GR (+) and (3) TF is a known down-stream target for indicated MAPK. N/A – literature data are not available. See complete references in Supplementary material #1. (**b**) Validation of the Protein-DNA array. The array data were validated by transient transfections of LNCaP-GR cells with corresponding Luciferase reporter vectors and RL reporter. Cells were treated with vehicle or FA (10^{-7} M × 24 h). The transfection data are presented as described in Figure 2e. The results of one representative experiment (three wells/experimental group) are shown as mean ± s.d.

SRF, STAT1/ISRE, STAT1 and NFATc factors with Luciferase under promoters containing the appropriate binding sites (similar or identical to ones in TF array) were transfected into LNCaP-GR cells (Figure 6b). We were able to confirm transrepression of all studied TFs, with the exception of NFAT-c.

To link our data on TF regulation by GR to the regulation of PC markers by GR/glucocorticoids, we screened promoter sequences of AMACR, hepsin and maspin (between positions −2000 and +1 from the transcription start sites) for the appropriate binding sites using online Transcription Element Search System

a



b **Hepsin**

TF	Position	Sequence
c-Ets-1	-1883	CCAGGAAG
	-1425	GCTTCCGGC
Elk-1	-1882	CAGGAAG
	-1169	GCAGGAACTG
C/EBPα	-1580	TGGGGCAAG
	-1296	AGCAAAC
	-797	CCCTCCTC
	-402	CCCTCCTC
CREB	-402	AGGnnnTACGCCA
ATF	-596	TGAGGTCAGGG
NFATc	-1383	TTTCC
c-Myc	-1419	CGCCCAGGTGGCC

AMACR

TF	Position	Sequence
c-Ets-1	-1112	GCAGGAAGCA
Elk-1	-1113	CAGGAAG
C/EBPα	-1323	ACTGGAAAT
	-1619	TTTGCAAAT
NFAT-c	-794	TTTTTCTCTT
GATA4	-883	AGATAT
AP-1/	-545	TGAGTTA
c-Fos/	-1642	GTGACTAA
c-Jun	-1491	TGACTAA
SRF	-1203	CCAAT

Figure 7 *In silico* analysis of putative TF binding sites in the promoters of PC markers. (**a**) Map of predicted TF binding sites of hepsin and AMACR promoters. 5′ Upstream promoter sequences relative to the transcription start sites between positions −2000 and +1 were analysed by online TESS. (**b**) Position and sequences of the predicted TF-DNA binding sites in PC marker promoters.

(TESS) (Schug and Overton, 2005). We found that promoters of hepsin and AMACR contained binding sites for TFs inhibited by GR including C/EBP-α, Ets-1, Elk-1, NFAT-c, SRF and GATA4 (Figure 7).

Discussion

Despite the wide use of glucocorticoids for PC treatment, the changes of GR expression during prostate tumorigenesis and its role in the prostate cells remain unknown. Here, we developed a comprehensive picture of GR expression during prostate tumorigenesis. We found that GR expression was decreased or absent in 70–85% of PCs compared to apparently normal prostate or BPH. We also revealed that the decrease in GR expression occurs early in prostate tumorigenesis, at the stage of HGPIN. The early loss of GR expression in prostate tumorigenesis resembles changes reported for estrogen receptor β, an inhibitor of prostate growth (Fixemer *et al*., 2003). In contrast, the expression of other steroid hormone receptors either remains stable, like AR, or is increased, like estrogen receptor α and progesterone receptor (Fixemer *et al*., 2003; Torlakovic *et al*., 2005). These results combined with our *in vitro* data discussed below strongly suggest a tumor suppressor role for GR in the prostate. Remarkably, the loss of GR was specific only for the epithelial compartment of PCs. In benign and malignant prostate specimens alike, the stromal cells showed predominant nuclear localization of GR (Figure 1 and Mohler *et al*., 1996), suggesting an important role of GR specifically in prostate epithelium. It will be important to understand the molecular mechanisms that underlie the decrease of GR expression in PC cells. Even though the regulation of GR expression has not been well studied, the recent data indicate that DNA methylation is one of the mechanisms of epigenetic regulation of GR expression (Weaver *et al*., 2005).

We evaluated the effects of restored GR signaling in LNCaP cells lacking endogenous GR on proliferation, differentiation and transformation. The inhibition of LNCaP-GR cell growth by FA correlated with the decreased Ki67 and Cyclin D1 expression, the increased expression of cell cycle inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, and the decreased expression in active c-Myc. Cell cycle-related proteins affected by glucocorticoids in LNCaP-GR cells are highly relevant to PC: cell cycle aberrations in PC are frequently linked to increased expression of cyclin D1 and other G1 phase cyclins, decrease in cell cycle inhibitor $p27^{Kip1}$, amplification and activation of c-Myc (reviewed by Quinn *et al*., 2005).

To evaluate the effect of GR signaling on LNCaP-GR cell differentiation, we analysed intermediate/late PC markers hepsin and maspin, and the early PC marker AMACR, all currently introduced for routine PC diagnostics (Dhanasekaran *et al*., 2001; Chen *et al*., 2003; Jiang *et al*., 2004). All these markers are important for PC growth, angiogenesis and metastases. AMACR and hepsin are protumorigenic (Zha *et al*., 2003; Klezovitch *et al*., 2004), whereas maspin is an established tumor suppressor in different types of epithelium, including prostate. Maspin blocks angiogenesis, growth

and invasion by PC cell *in vitro* and *in vivo* (reviewed by Q6 Schaefer and Zhang, 2003). We showed that GR-mediated signaling promotes differentiated state in PC cells where potential oncogenes hepsin and AMACR are downregulated and the expression of tumor suppressor maspin is increased. We also showed dramatic decrease of transformation reflected by the loss of anchorage-independent growth. In summary, we for the first time demonstrated overall normalization of PC cell phenotype by GR signaling.

Finally, we performed fine dissection of the mechanisms underlying GR antitumor activity in PC cells. One important mode of gene regulation by GR, transrepression, is in most cases mediated by direct interaction between GR and other TFs or by the crosstalk between GR and other signaling pathways, especially MAPKs (Kassel *et al*., 2001; Schacke *et al*., 2002; Bruna *et al*., 2003; De Bosscher *et al*., 2003; Necela and Cidlowski, 2004).

MAPK-mediated signaling is crucial for proliferation and survival of tumor cells (Greenberg *et al*., 2002; Ricote *et al*., 2006). Although changes in activation of specific MAPKs during prostate tumorigenesis are complex, nuclear expression of activated Erk and p38 and the level of phosphorylation of their targets Elk-1 and ATF-2 are frequently increased in PCs (Ricote *et al*., 2006; Gioeli *et al*., 2006). We found that activation of GR signaling blocked the activity of four major MAPKs: p38, JNK/SAPK, Mek1/2 and Erk1/2. These data are in line with the observations that glucocorticoids suppress MAPKs activity in some cell types. One of the mechanisms of GR inhibition of MAPK involves increased expression of MKP1 (Kassel *et al*., 2001), a primary glucocorticoid-responsive gene (Wu *et al*., 2004). We also found the increased MPK1 expression in GR-positive cells treated with glucocorticoids. Our experiments also revealed an additional level of MAPK regulation by glucocorticoids, via post-transcriptional decrease of the total MAPK protein amount. Overall our results indicate that MAPKs are tightly regulated by GR/glucocorticoids in prostate cells.

Further, we showed that among numerous TFs whose activity was altered by GR in LNCaP cells, more than 85% were downregulated upon GR activation. Many of those, including AP-1, SRF, Ets-1, Elk-1, STAT1/ISRE, ATF2, C/EBPα, GATA4, EGR1 and PAX6 are recognized MAPK targets (references in Figure 6a and Supplementary material #1). Thus, their downregulation is an obvious consequence of the MAPK blockade by glucocorticoids. However, without further studies, we cannot rule out other mechanisms including the diminished expression of those TFs or their direct interaction with GR. Importantly, TFs repressed by GR contribute to the different steps of prostate tumorigenesis (references in Figure 6a and Supplementary material #1), and may control the expression of PC markers and differentiation of PC cells (Peterziel *et al*., 1999; Q7 Grossmann *et al*., 2001).

In summary, our results suggest that GR signaling has an antitumor effect in prostate cells, and that glucocorticoid treatment of patients at early stages of prostate tumor development such as HGPIN, when PC cells still express GR, may result in the inhibition of PC growth and normalization of PC cell phenotype. In the future, it will be important to extend our studies, and to evaluate the GR expression in prostate at the stage of HRPC and in PC metastatic lesions. In any case, the changes in GR expression should be taken into consideration to design the optimal time regimens for PC patient treatment with these steroid hormones and to enhance the clinical benefit of glucocorticoid therapy.

Materials and methods

Cell cultures and treatments

LNCaP cells (American Tissue Culture Collection, Rockville, MD, USA) were cultured in Roswell Park Memorial Institute 1640 medium (Gibco BRL Life Technologies, Rockville, MD, USA) with 10% FBS (HyClone, Logan, UT, USA), sodium pyruvate (10 mM), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (10 mM) and antibiotics (Gibco BRL Life Technologies, Rockville, MD, USA) (referred thereafter as complete medium). The cells were treated with 10^{-9}–10^{-6} M FA (Sigma, Saint Louis, MO, USA), TNFα (10 ng/ml), IL-1 (1 μg/ml) and EGF (1–100 ng/ml) (all from BioSource Inc., Camarillo, CA, USA) where indicated.

Generation of LNCaP-GR cells

To generate LNCaP cells stably expressing rat GR cDNA (kindly provided by Dr M Beato, Philipps-Universitat, Marburg, Germany) tagged with V5 at the C-terminus, we used lentiviral system (Invitrogen Corp., Carlsbad, CA, USA). For selection of GR-positive clones, 6 μg/μl blasticidin was applied. For easier tracking, a second line of LNCaP cells was co-infected with CMV.GR-V5tag and YFP lentiviruses, and the cells containing YFP were selected by sorting. Control cell lines were established by infecting LNCaP cells with either the empty lentivirus (LNCaP-V) or the lentivirus-expressing YFP (LNCaP-YFP).

Western blot analysis

Whole-cell protein extracts were prepared using radioimmunoprecipitation assay buffer as described elsewhere (Rosenberg, 1996), resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% gels, transferred to nitrocellulose membranes (BioRad, Hercules, CA, USA), incubated with primary Abs (see Supplementary material #2) followed by peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Ig)G secondary Abs (Cell Signaling Technology, Beverly, MA, USA) and ECL reagent (Amersham Pharmacia Biotech, Sweden) for the band visualization. To verify equal loading and adequate transfer, the membranes were probed with anti-actin and/or anti-β-tubulin Abs (Santa Cruz Biotechnology, Pasadena, CA, USA). To quantify the signals, images were scanned and digitized using ImageJ software (NIH, Bethesda, MD, USA).

Transient transfections and Luciferase assay

PC cells at 70% confluence were transfected with reporter Luciferase plasmids (see the list of reporter plasmids in the Supplementary material #3) in 24-well plates using Effectene reagent (Qiagen Inc., Valencia, CA, USA). Each well totally contained 0.2 μg of the plasmid DNA. All experimental and control groups contained at least three wells. The cells were harvested 36 h after transfection and Luciferase activity was

measured using commercial Luciferase assay (Promega Corp., San Luis Obispo, CA, USA) on a TD20/20 Turner luminometer (Turner Design Instruments, Sunnyvale, CA, USA). When necessary, the cells were pretreated with 10^{-9}–10^{-6} M FA or vehicle (0.1% ethanol) for 2 days before transfections. The transfection efficacy was normalized using *Renilla* Luciferase (RL) under minimal promoter (Promega, Madison, WI, USA) to equalize for the transfections efficiency.

RT–PCR

A two-step RT–PCR reaction using reverse transcriptase murine leukemia virus-RT, random primers and PCR-Supermix (both from Invitrogen Corp., Carlsbad, CA, USA) with appropriate PCR primers was performed using total RNA isolated by the RNAeasy kit (Qiagen Inc., Valencia, CA, USA). The PCR primers (see the primer sequences in the Supplementary material #4) were designed using the PrimerBank database (http://pga.mgh.harvard.edu/primerbank/), RTPrimerDB Real Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb/index.php) and Vector NTI software (Invitrogen Corp., Carlsbad, CA, USA).

PCR products were run on 1.5% agarose gels, the actual amount of PCR product was measured by Agilent 2001 Bioanalyzer and normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR product. The quantitative data are presented as the ratio of GAPDH-normalized amount of PCR product in FA-treated vs vehicle-treated cells.

Proliferation assay

The proliferation was measured by direct cell counts, or for the YFP-expressing cells fluorescence was measured by a Victor plate reader (Perkin-Elmer, Boston, MA, USA) at 436 nm excitation, and 480 nm emission wavelengths. For both tests, the cells plated at 10^4 cells/well onto 12-well plates were cultured in complete media with 6 μg/ml blasticidin in the presence of FA, EGF or vehicle (0.1% ethanol) for 1–12 days. Each experimental and control group consisted of three wells.

Colony formation assay in soft agar

Q8 The modification of previously described standard assay (Li and Johnson, 1998) was used. Briefly, the cells were trypsinized, washed in complete medium, resuspended in the medium with 0.6% agar and plated over the pre-formed agar underlayers (1% agar in complete medium) in 12-well plates (10^4 cells in 350 μl/well). After 2 and 4 weeks, the colonies were analysed using Zeiss fluorescent inverted microscope AxioVert. Each experimental and control group consisted of six wells.

Immunostaining of prostate tissues and cell cultures

Details of tissue collection procedure, immunostaining of cell cultures and tissue samples, and morphological evaluation are described in Supplementary material #5. Tissues were obtained from two cohorts of consented untreated patients (aged 40–82 years) by TURP (transurethral prostatic resection) or radical prostatectomy. Immunostaining of paraffin-embedded sections of formalin-fixed prostate samples was performed using primary mouse monoclonal anti-GR Abs (Novocastra, Norwell, MA, USA) followed by secondary anti-mouse IgG-reagent provided in the diaminobenzidine chromogen-based Envision + System-HRP kit (DakoCytomation, Carpinteria, CA, USA) and counterstained in Mayer's hematoxylin. The number of prostate epithelial cells with nuclear GR signal was evaluated by + to + + + scoring.

Immunostaining of cell cultures was performed on sterile coverslips. Cells were fixed, permeabilized and incubated with primary Abs (overnight at 4°C) followed by anti-rabbit donkey fluorescein isothiocyanate-conjugated and/or anti-mouse donkey Cy-3-conjugated secondary Abs (both from Jackson Immunoresearch, West Grove, PA, USA) and application of 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA, USA) to identify the nuclei.

Transcription factor protein/DNA arrays

To simultaneously evaluate the activity of multiple TFs, we used Combo-Array version of TranSignal protein/DNA interaction array (Panomics Inc., Fremont, CA, USA) containing probes for binding sites for over 300 TFs (for detailed description see Jiang *et al*., 2004 and Supplementary material #6). The experiment was repeated three times. The differences in signal between FA- and vehicle-treated samples $\geqslant 2$ were considered statistically and biologically significant if they were revealed in all three experiments.

In silico *analysis of TF binding sites*

In silico promoter analysis of hepsin, maspin and AMACR was performed using 5′ upstream promoter sequences between positions −2000 and + 1 from the transcription start sites. The online Transcription Element Searching System TESS was used (Schug and Overton, 2005). Only TF binding sites displaying no variability from canonical sequences were selected for the analysis.

Statistical analysis

All experiments were repeated at least three times. Mean and s.e. values were calculated using Microsoft Excel software and compared using paired Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

Abbreviations

AMACR, alpha-methylacyl-CoA racemase; AP-1, activator protein 1; BPH, benign prostatic hyperplasia; Erk1/2, extracellular signal-regulated kinase 1 and 2; FA, fluocinolone acetonide; HGPIN, high-grade prostatic intraepithelial neoplasia; HRPC, hormone refractory prostate carcinoma; MAPKs, mitogen-activated protein kinases; Mek1/2, dual specificity mitogen-activated protein kinase 1 and 2; NF-κB, nuclear factor kappa-B; PC, prostate carcinoma; PIN, prostatic intraepithelial neoplasia; SAPK/JUNK, stress-activated protein kinase/c-Jun-N-terminal kinase; TF, transcription factor; YFP, yellow fluorescent protein.

Acknowledgements

This work was supported by the DOD prostate grant DAMD17-03-1-0522 and the Northwestern University Prostate SPORE Developmental Project (to IB). We thank Drs KR Yamamoto (University of California, San Francisco, CA, USA), WC Greene (Gladstone Institute for Virology and Immunology, University of California, San Francisco, CA, USA), DJ Klumpp, (Northwestern University, Chicago IL, USA), M Beato, (Philipps-Universitat, Marburg, Germany) for their generous gift of plasmids. We are grateful to Drs P Stern, J Pelling and O Volpert (Northwestern University, Chicago, IL, USA) for fruitful discussions of our work.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).